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Interaction of homologues of Hsp70 and Cpn60 with ferredoxin-NADP$^+$ reductase upon its import into chloroplasts

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A homologue of the 70-kDa heat-shock protein (Hsp70) was purified from pumpkin chloroplasts. The molecular mass of the purified protein was approximately 75 kDa and its N-terminal amino acid sequence was very similar to those of homologues of Hsp70 from bacterial cells and from the mitochondrial matrix and stroma of pea chloroplasts. The purified homologue of Hsp70 was found in the stroma of chloroplasts. To investigate the role(s) of the homologue of Hsp70 in the chloroplast stroma, we examined the possibility that the homologue of Hsp70 might interact with newly imported proteins to assist in their maturation (for example, in their folding and assembly). Ferredoxin NADP$^+$ reductase (FNR) imported into chloroplasts in vitro could be immunoprecipitated with antisera raised against the homologue of Hsp70 from pumpkin chloroplasts and against GroEL from *Escherichia coli*, which is a bacterial homologue of chaperonin 60 (Cpn60), in an ATP-dependent manner, an indication that newly imported FNR interacts physically with homologues of Hsp70 and Cpn60 in chloroplasts. Time-course analysis of the import of FNR showed that imported FNR interacts transiently with the homologue of Hsp70 and that the association of FNR with the homologue of Hsp70 precedes that with the homologue of Cpn60. These results suggest that homologues of Hsp70 and Cpn60 in chloroplasts might sequentially assist in the maturation of newly imported FNR in an ATP-dependent manner.

Chloroplast; 70-kDa Heat-shock protein (Hsp70); Chaperonin 60 (Cpn60); Ferredoxin NADP$^+$ reductase (FNR); Pumpkin (*Cucurbita* sp.); Pea (*Pisum sativum*)

1. INTRODUCTION

Molecular chaperones are a class of cellular proteins that play roles in the transport, folding and assembly of certain other polypeptides but are not components of the final oligomeric structures [1]. The members of the family of 70-kDa heat-shock proteins (Hsp70) are proposed to be molecular chaperones. These Hsp70s have highly conserved amino acid sequences and have been found in bacteria and in subcellular compartments of eukaryotic cells [2]. It has been reported that cytosolic homologues of Hsp70 can facilitate translocation of proteins across the ER and mitochondrial membranes [3,4], and that homologues of Hsp70 in the ER and mitochondria appear to assist in the translocation of proteins across these membranes from the trans-sides [5,6]. There is also evidence that they can support the folding and assembly of newly translocated proteins inside these structures [2,6]. The chloroplast homologues of Hsp70 have been detected immunologically in the outer envelope membranes and stroma of chloroplasts [7] and, recently, cDNAs encoding a homologue of Hsp70 in the outer envelope membranes of spinach chloroplasts [8] and a homologue of Hsp70 in the stroma of pea chloroplasts [9] were isolated and the primary structures of the proteins were deduced. A recent study suggested that a homologue of Hsp70 in the stroma of barley chloroplasts might be involved in the integration of the apoprotein precursor of the major light-harvesting complex of photosystem II into thylakoid membranes [10]. However, it remains unclear whether or not the stromal homologue of Hsp70 is involved not only in the integration of the protein into the thylakoid membrane but also in the translocation of proteins across the envelope membranes, the folding and the assembly of proteins. In this report, we present evidence that a protein, upon its import into chloroplasts, interacts physically with homologues of Hsp70 and chaperonin 60 (Cpn60), the latter protein being another molecular chaperone that is homologous to GroEL of *Escherichia coli*.

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Abbreviations: ER, endoplasmic reticulum; FNR, ferredoxin NADP$^+$ reductase; pFNR, precursor to FNR; Hsp70, 70-kDa heat-shock protein; Cpn60, chaperonin 60.

2. MATERIALS AND METHODS

2.1. *Plant materials*

Seeds of pumpkin (*Cucurbita* sp. Kurokawa Amakuri Nankin) and pea (*Pisum sativum* cv. Alaska) were soaked in tap water overnight and seedlings were cultured for 4 days in darkness and subsequently for 4–6 days in white light.

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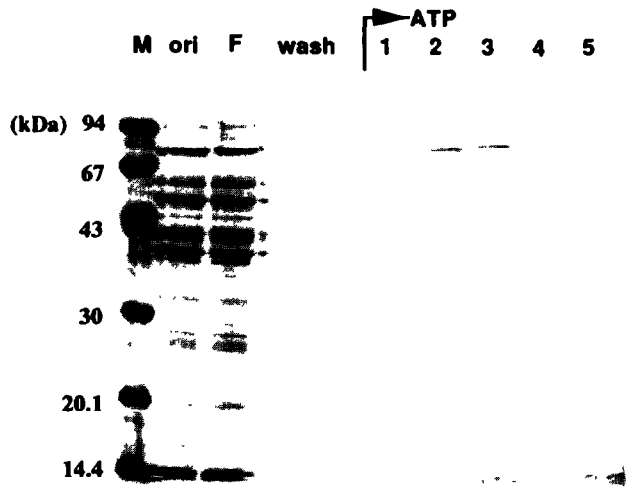

Fig. 1. Purification of a homologue of Hsp70 from isolated pumpkin chloroplasts. Solubilized proteins from pumpkin chloroplasts were directly applied to an ATP-agarose column. Elution was performed with 5 mM ATP after washing with excess high-salt buffer. Solubilized proteins from pumpkin chloroplasts (ori), the flow-through fraction (F), fractions washed from the column (wash) and fractions eluted with ATP (1,2,3,4 and 5) were subjected to SDS-PAGE and subsequent staining with Coomassie blue R-250. M, marker proteins; phosphorylase *b* (94 kDa); bovine serum albumin (67 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa); soybean trypsin inhibitor (20 kDa); and α-lactoalbumin (14.4 kDa).

2.2. *Preparation of chloroplasts, stroma, thylakoids and envelope membranes*

Intact chloroplasts were prepared from homogenates of green cotyledons of pumpkin seedlings and green leaves of pea seedlings by a combination of differential centrifugation and Percoll step-gradient centrifugation [11]. Stroma, thylakoids and envelope membranes were obtained by fractionation of intact chloroplasts by the method of Smeekens et al. [12].

2.3. *Purification of chloroplast Hsp70 and determination of its N-terminal amino acid sequence*

Intact chloroplasts were solubilized in buffer A [20 mM Tris-acetate (pH 7.4), 20 mM NaCl, 5 mM $MgCl_2$, 15 mM β-mercaptoethanol and 0.1% Triton X-100]. After centrifugation at 100,000 × *g*, solubilized proteins were applied directly to an ATP-agarose column. The column was then washed with excess buffer A and with buffer A that contained 1 M NaCl. Then proteins were eluted with buffer A that contained 5 mM ATP. The eluted fraction contained one major protein with a molecular mass of 75 kDa. The N-terminal amino acid sequence of the purified 75-kDa protein was determined by the procedure of Matsudaira [13].

2.4. *Preparation of radiolabeled pFNR*

cDNA encoding the precursor to ferredoxin $NADP^+$ reductase (pFNR) was synthesized from total RNA from green leaves of pea seedlings by use of a sequence-specific primer [14] and amplification by the polymerase chain reaction in which *Taq* DNA polymerase was used. ^{35}S-labeled pFNR was prepared from cDNA that encoded pFNR as described [15].

2.5. *Import of pFNR into chloroplasts*

Import of pFNR was assayed in 1 ml of import buffer [50 mM HEPES-KOH (pH 8.0), 0.33 M sorbitol] that contained chloroplasts (the equivalent of 1 mg of chlorophyll) and radiolabeled pFNR. Import reactions were allowed to proceed at 25°C in the light.

2.6. *Immunoprecipitation*

Immunoprecipitation was carried out basically by the same method as described previously [16]. Chloroplast pellets obtained from aliquots of the import reaction mixture were solubilized in 1 ml of buffer B [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Triton X-100] that contained 5 mM $MgCl_2$, 10% (v/v) glycerol and 0.2 mM NADPH with 2.5 U/ml apyrase (Sigma, USA) or 2 mM ATP. Samples were incubated for 15 min at 4°C and then centrifuged at 4°C for 10 min at 15,000 × *g* to remove insoluble materials. Aliquots of the supernatant were mixed with 20 μl of a pre-immune serum or antiserum raised against the purified homologue of Hsp70 from pumpkin chloroplasts (anti-chl Hsp70) or against GroEL from *Escherichia coli* (anti-Cpn60) [17], as well as with 100 μl of a 1:1 (v/v) slurry of protein A-Sepharose CL4B (Pharmacia, Sweden) in buffer B. The samples were incubated

Pumpkin Chloroplast Hsp70	QKVVGIDLGTTNSAVAAMEGGKPTIVTNAEGQRT
Pea chloroplast stroma Hsp70 (CSS1)	E----------I--------
***Synechocystis* sp. PCC 6803 DnaK**	G----------C--V-------VIA----F--
***E.coli* DnaK**	G-II---------C--I-D-TT-RVLE----D--
Pea Mitochondrial Hsp70 (PHSP1)	ND-I---------C-SV---KN-KVIE-S--A--
Yeast Mitochondrial Hsp70 (SSC1)	GS-I----------I---KV-K-IE----S--
Yeast ER Hsp70 (KAR2)	GT-I-------Y-C--V-KN--TE-LA-EQ-N-I
Yeast Cytosol Hsp70 (SSA1)	S-A--------Y-C--HFANDRVD-IA-DQ-N--
Spinach Chloroplast Envelope Hsp70 (SCE70)	GPAI-------Y-R-GVWQHDRVE-IA-DQ-N--
Possible ATP-Binding Site	••••••••••
(Nucleotide β-Phosphate-Binding Site)	IXIDXGTTXXC L L SS R V V GG K C

Fig. 2. Comparison of the N-terminal amino acid sequence of a homologue of Hsp70 in pumpkin chloroplasts with those of other homologues of Hsp70. N-terminal amino acid sequences of the mature polypeptides of homologues of Hsp70 from pumpkin chloroplasts (this work); pea chloroplasts [9]; *Synechocystis* sp. PCC 6803 [26]; *E. coli* [27]; pea mitochondria [28]; mitochondria [29] ER [30,31]; and cytosol [32] of *Saccharomyces cerevisiae*; and spinach chloroplast envelopes [8] are arranged in terms of maximum similarity The symbol (-) indicates that an amino acid residue is identical to that of the homologue of Hsp70 from pumpkin chloroplasts. Underlining indicates possible ATP-binding sites [18].

at least for 4 h with gentle agitation. The protein A-Sepharose beads were collected and washed five times with 1-ml aliquots of buffer B. The bound immune complexes were then dissociated in SDS-PAGE sample buffer. Each sample was subjected to SDS-PAGE and gels were analyzed with a Bio-Imaging Analyzer (Fujix BAS2000, Fuji Photo Film, Tokyo).

3. RESULTS AND DISCUSSION

All members of the Hsp70 family that have been characterized to date have weak ATPase activity and can bind ATP [2]. A protein with a molecular mass of approximately 75 kDa was purified by affinity chromatography on an ATP-agarose column from a soluble extract of pumpkin chloroplasts (Fig. 1). In order to confirm that the purified 75-kDa protein was a homologue of Hsp70, we determined its N-terminal amino acid sequence. Fig. 2 shows that the N-terminal amino acid sequence is very similar to those of homologues of Hsp70 from various sources, in particular at their putative ATP-binding sites, as previously characterized elsewhere [18]. However, the amino acid sequence outside the possible ATP-binding site exhibited much greater similarity to those of bacterial and mitochondrial homologues of Hsp70, as well as to that of a homologue of Hsp70 in the stroma of pea chloroplasts, than to those of homologues of Hsp70 in the cytosol and ER. These results strongly suggest that the purified protein was a homologue of Hsp70 that is present in pumpkin chloroplasts and is a bacterial type of Hsp70. An antiserum raised against the purified protein (anti-chl Hsp70) specifically recognized proteins with a molecular mass of approximately 75 kDa in crude extracts of chloroplasts isolated not only from pumpkin cotyledons but also

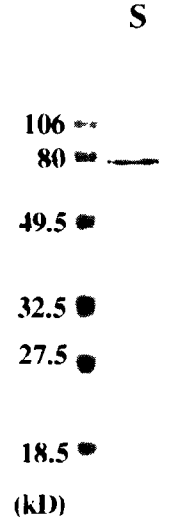

Fig. 3. Suborganellar localization of a homologue of Hsp70. Fractions containing stroma (S), thylakoids (T) and envelope membranes (EM) were separated from chloroplasts isolated from pumpkin cotyledons and were then subjected to SDS-PAGE and subsequent immunoblotting with anti-chl Hsp70 serum.

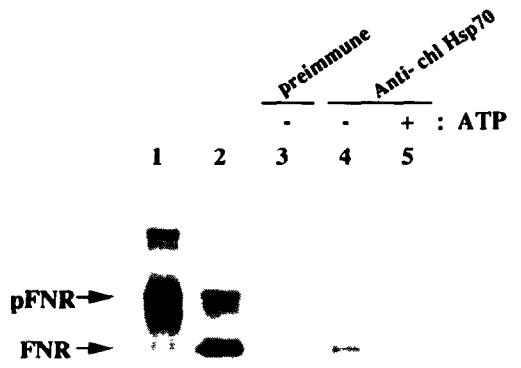

Fig. 4. ATP-dependent interaction of a homologue of Hsp70 with newly imported FNR. ^{35}S-labeled precursor to ferredoxin NADP$^+$ reductase (pFNR), synthesized in a rabbit reticulocyte lysate translation system using an in vitro transcript of the cDNA, was incubated with isolated pea chloroplasts at 25°C in the light. After 20 min, reaction mixtures were diluted tenfold with ice-cold import buffer that contained 5 μg/ml nigericin. Chloroplasts were recovered by centrifugation and washed once. Chloroplast pellets were solubilized in buffer A (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Triton X-100) that contained 5 mM $MgCl_2$ and 10% (v/v) glycerol with 2.5 U/ml apyrase (lanes 3,4) or 2 mM ATP (lane 5). Samples were incubated for 15 min at 4°C and then centrifuged to remove insoluble materials. Supernatants were subjected to immunoprecipitation with anti-chl Hsp70 serum (lanes 4,5) or a preimmune serum (lane 3). Lane 1, translation products of pFNR-specific RNA; lane 2, chloroplasts recovered after import reaction of pFNR into chloroplasts. Each sample was subjected to SDS-PAGE and the gel was analyzed with a Bio-Imaging Analyzer (Fujix BAS2000; Fuji Photo Film, Tokyo).

from spinach and pea leaves (data not shown). We examined the sub-organellar localization of the homologue of Hsp70 in pumpkin chloroplasts. Immunoblot analysis showed that the homologue of Hsp70 was located specifically in the stromal fraction of chloroplasts (Fig. 3). This result indicates that the purified homologue of Hsp70 was a stromal protein.

To examine whether or not the homologue of Hsp70 in the chloroplast stroma interacts with proteins upon their import into chloroplasts, we constructed a system for monitoring import of proteins into chloroplasts in vitro. The precursor to FNR was imported into chloroplasts and was processed to a mature form in vitro (Fig. 4, lanes 1,2). Anti-chl Hsp70 serum was employed to analyze the possible interaction between the homologue of Hsp70 and FNR that had been newly imported into chloroplasts. As shown in Fig. 4 (lanes 4,5) newly imported FNR was immunoprecipitated with anti-chl Hsp70 when levels of ATP were reduced by apyrase. This result strongly indicates that newly imported FNR interacts physically with the stromal homologue of Hsp70. A homologue of Cpn60 in the chloroplast stroma was proposed to be involved in a process of assembly of ribulose bisphosphate carboxylase-oxygenase (Rubisco), because newly synthesized large subunit of Rubisco forms a complex with the homologue of

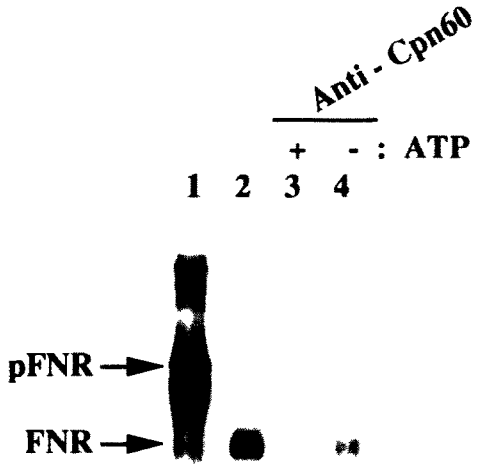

Fig. 5 ATP-dependent interaction of a homologue of Cpn60 with newly imported FNR. ^{35}S-labeled pFNR, synthesized in a rabbit reticulocyte lysate translation system using an in vitro transcript of the cDNA, was incubated with isolated pea chloroplasts at 25°C in the light After 20 min, reaction mixtures were diluted tenfold with icecold import buffer that contained 5 μg/ml nigericin. Chloroplasts were recovered by centrifugation and washed once. Chloroplast pellets were solubilized in buffer A (50 mM Tris-HCL (pH 8 0), 150 mM NaCL, 0.1%Triton X-100) that contained 5 mM $MgCl_2$ and 10% (v/v) glycerol with 2.5 U/ml apyrase (lane 4) or 2 mM ATP (lane 3). Samples were incubated for 15 min at 4°C and then centrifuged to remove insoluble materials. Supernatants were subjected to immunoprecipitation with anti-Cpn60 (GroEL) serum (lanes 3,4). Lane 1, translation products of pFNR-specific RNA. Lane 2, chloroplasts recovered after import reaction of pFNR into chloroplasts. Each sample was analyzed as described in the legend to Fig. 4.

Cpn60 before assembly into holoenzyme [19]. It was reported that several proteins newly imported into chloroplasts co-migrate with the homologue of Cpn60 on non-denaturing gel electrophoresis [20] and that in the

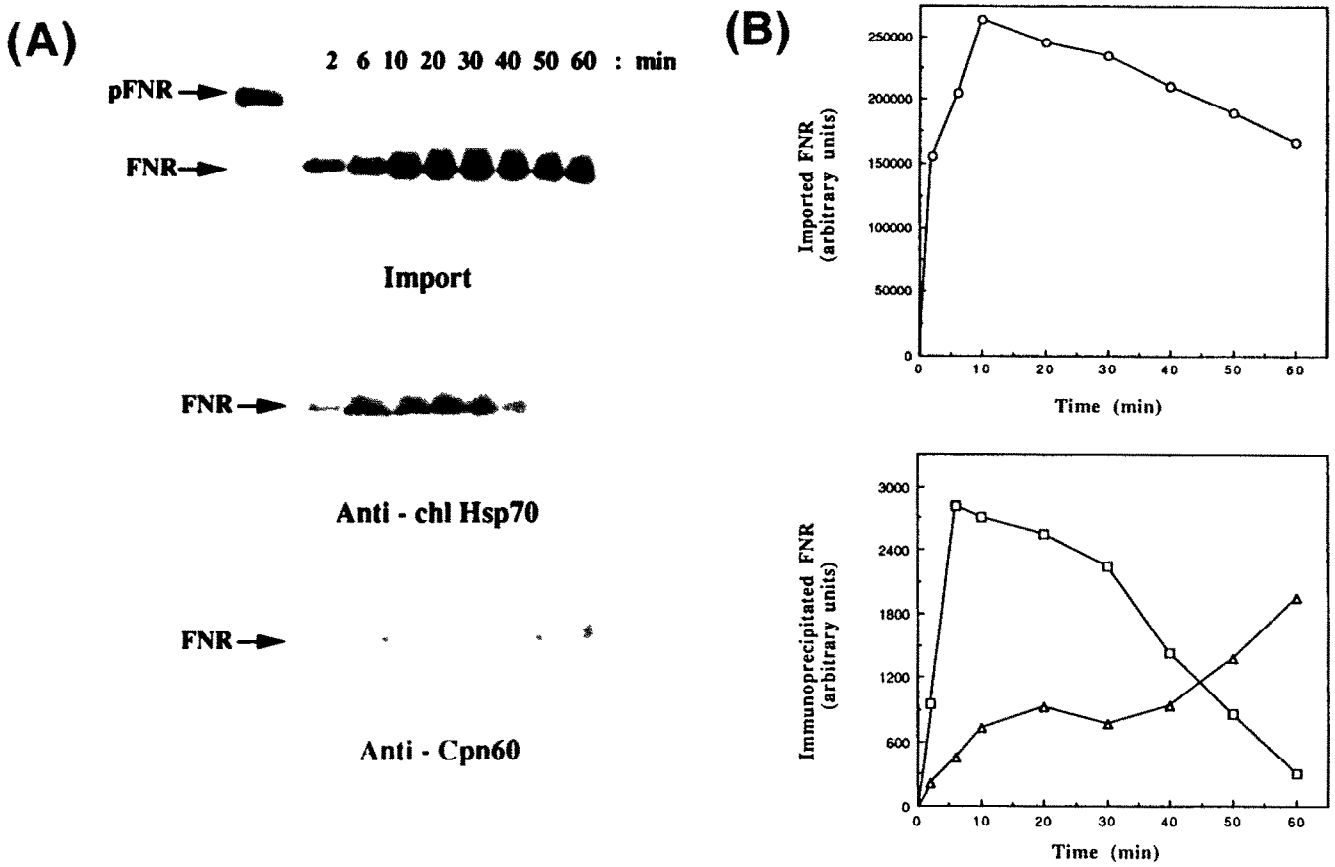

Fig. 6. Time course of binding of FNR to homologues of Hsp70 and Cpn60. (A) ^{35}S-labeled pFNR was incubated with isolated pea chloroplasts at 25°C in the light. At the indicated times, aliquots were removed and incubated for 10 min at 0°C with 7.5 μg/ml proteinase K. Chloroplasts were recovered by centrifugation through 40% Percoll in import buffer and washed twice with import buffer that contained 5 μg/ml nigericin. Chloroplast pellets were solubilized in buffer A (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Triton X-100) that contained 0.2 mM NADPH, 10% (v/v) glycerol and 2.5 U/ml apyrase. Samples were incubated for 15 min at 4°C and then centrifuged to remove insoluble materials. Immunoprecipitation was performed with anti-chl Hsp70 serum and anti-Cpn60 (GroEL) serum. Each sample was analyzed as described in the legend to Fig. 4. (B) Immunoprecipitated FNR with anti-chl Hsp70 (Immunoprecipitated FNR, □), and with anti-Cpn60 (Immunoprecipitated FNR, △) as well as the total amount of mature form of FNR (Imported FNR, ○) were microelectronically with a Bio-Imaging Analyzer (Fujix BAS2000; Fuji Photo Film, Tokyo) and multiplied by the corresponding factor to account for the amounts used for every set of samples. Numbers on the vertical axis represent arbitrary units.

case of mitochondria of yeast, newly imported proteins interact sequentially and ATP-dependently with homologues of Hsp70 and Cpn60 in the matrix [21]. We previously described an antiserum raised against Cpn60 (GroEL) from *Escherichia coli* (anti-Cpn60), which specifically recognizes Cpn60 in the mitochondria and plastids of plant cells [17]. We examined, by immunoprecipitation with the antiserum, whether a homologue of Cpn60 in chloroplasts might also interact with newly imported FNR. Newly imported FNR was co-immunoprecipitated by anti-Cpn60 when levels of ATP were reduced by apyrase (Fig. 5). Time-course analysis of the import of FNR showed that imported FNR interacted transiently with the homologue of Hsp70 and that the association of FNR with the homologue of Hsp70 preceded that with the homologue of Cpn60 (Fig. 6A,B). However, it must be pointed out that FNR associated with the homologue of Cpn60 was accumulated during incubation time as shown in Fig. 6B. This is quite different from transient association of the homologue of Cpn60 in mitochondria of yeast [21]. These results clearly show that homologues of Hsp70 and Cpn60 in the stroma of chloroplasts interact with newly imported FNR in different ways. Analysis in vitro of the effects of bacterial molecular chaperones on the folding of bovine rhodanase that had been denatured in vitro demonstrated that interaction of DnaK, a bacterial homologue of Hsp70, with rhodanase precedes that of GroEL, a bacterial homologue of Cpn60, in the generation of the native state of rhodanase [22]. Our time-course analysis supports this result, namely that a homologue of Hsp70 interacts with imported FNR before a homologue of Cpn60 does so. These results support the hypothesis that the interaction of the homologues of Hsp70 and Cpn60 with newly imported FNR in chloroplasts may be involved in the conversion of FNR to its active form. In fact, a recent study of the expression of pea FNR in *E. coli* revealed that folding and assembly of an enzymatically competent holoenzyme of FNR required bacterial homologues of Cpn60 and chaperonin 10, namely, products of the *groE* genes, GroEL and GroES [23]. Further analysis using other chloroplast proteins as substrates for these molecular chaperones may help us to clarify the function of the homologues of Hsp70 and Cpn60 in translocation, folding and assembly of proteins in chloroplasts.

In higher plants, FNR is found in at least two forms: a stromal FNR that can be released from chloroplasts by osmotic shock; and a membrane-bound FNR that remains more tightly bound to the thylakoid membrane [24]. Accumulating evidence suggests that the membrane-bound form is physiologically significant [25]. A study of the function of molecular chaperones in the binding of FNR to thylakoid membrane is in progress.

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