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Identification, characterization, and DNA sequence of a functional "double" groES-like chaperonin from chloroplasts of higher plants

(molecular chaperones/stress proteins/groEL/protein import/protein folding)

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ABSTRACT **Chloroplasts of higher plants contain a nuclear-encoded protein that is a functional homolog of the *Escherichia coli* chaperonin 10 (cpn10; also known as groES). In pea (*Pisum sativum*), chloroplast cpn10 was identified by its ability to (*i*) assist bacterial chaperonin 60 (cpn60; also known as groEL) in the ATP-dependent refolding of chemically denatured ribulose-1,5-bisphosphate carboxylase and (*ii*) form a stable complex with bacterial cpn60 in the presence of Mg·ATP. The subunit size of the pea protein is ≈24 kDa—about twice the size of bacterial cpn10. A cDNA encoding a spinach (*Spinacea oleracea*) chloroplast cpn10 was isolated, sequenced, and expressed *in vitro*. The spinach protein is synthesized as a higher molecular mass precursor and has a typical chloroplast transit peptide. Surprisingly, however, attached to the transit peptide is a single protein, comprised of two distinct cpn10 molecules in tandem. Moreover, both halves of this "double" cpn10 are highly conserved at a number of residues that are present in all cpn10s that have been examined. Upon import into chloroplasts the spinach cpn10 precursor is processed to its mature form of ≈24 kDa. N-terminal amino acid sequence analysis reveals that the *mature* pea and spinach cpn10 are identical at 13 of 21 residues.**

Molecular chaperones play a vital role in protein folding and protein translocation in eukaryotic and prokaryotic cells (for a recent review see ref. 1). Among the best-studied molecular chaperones are the "chaperonin" proteins (2), which in *Escherichia coli* consist of two family members—groEL or chaperonin 60 (cpn60) and groES or chaperonin 10 (cpn10)—that are encoded by a common operon (3). Both proteins are essential for full chaperonin function *in vivo* (4–7). The structure of groEL is a double toroid, each ring consisting of seven identical 60-kDa subunits (8), whereas groES is thought to be a seven-membered ring of identical 10-kDa subunits (9). In the presence of ATP (9, 10), the two chaperonin components form a stable complex with each other, resulting in an inhibition of the "uncoupled" ATPase of groEL. Electron micrographs of isolated groEL/groES complexes suggest that the groEL "double donut" interacts with a single heptameric ring of groES (11), consistent with the stoichiometry reported for the chaperonin-assisted refolding of dihydrofolate reductase (12).

Recent studies have shown that purified groEL and groES can assist in the *in vitro* refolding of various proteins that are structurally unrelated in their native states (10, 12–17). Based on "order of addition experiments" (10, 13) with the dimeric ribulose-1,5-bisphosphate carboxylase (Rubisco) of *Rhodospirillum rubrum*, a general two-step mechanism was proposed. In the first step, cpn60 captures a labile Rubisco folding intermediate with which it forms a stable binary complex. While sequestered on cpn60, nonnative Rubisco cannot refold to its native state but is protected from aggregation it would otherwise experience. In the second step, the cpn60/Rubisco complex is discharged in some unknown manner that ultimately yields catalytically active Rubisco. Depending on the target protein and the experimental conditions, this step requires the participation of cpn10 and a K^+-dependent hydrolysis of Mg·ATP (10, 12, 13). The available evidence suggests that cpn10 is a "coupling factor" (10) that plays an important role in preventing the *premature* release of partially folded proteins from their complexes with groEL—a situation that might occur in the presence of ATP alone (1, 12, 18).

Mitochondria (19, 20) and chloroplasts (2, 21) also contain cpn60. Recently, a purified mitochondrial cpn60 (mt-cpn60) from Chinese hamster ovary cells was shown to assist in the *in vitro* refolding of denatured Rubisco (22). As anticipated, this reaction required Mg·ATP, K^+ ions, and the participation of a mitochondrial cpn10 (mt-cpn10) that was previously purified from bovine liver (23). Surprisingly, bacterial cpn10 (b-cpn10) could not substitute for mt-cpn10 in this reaction, despite the fact that the latter is functionally compatible with bacterial cpn60 (b-cpn60) (23). In any event, it is now clear that the mitochondrial chaperonin system, like that of prokaryotes, requires two distinct protein components that interact with each other in the presence of ATP (22).

Little is known about the functional properties of the chloroplast chaperonins. Purified preparations of chloroplast cpn60 (ch-cpn60) contain two distinct polypeptides (α and β) that are present in equal amounts (2, 24). Both proteins are typical cpn60 family members; however, they are highly divergent, exhibiting only 50% identity to each other (25). The organization of α and β subunits within the native ch-cpn60 tetradecamers is not known. Finally, although it is suspected that a chloroplast cpn10 (ch-cpn10) probably exists (13, 23), no such protein has been described. Here we report the cDNA sequence¶ and biochemical characterization of a functional cpn10 homolog that is present in higher plant chloroplasts. Most intriguingly, the subunit molecular mass of this protein is about twice the size of bacterial or mitochondrial cpn10. Primary amino acid sequence analysis reveals that the chloroplast cochaperonin is a "double" cpn10 molecule that has arisen through either gene duplication or fusion of two different ancestral cpn10 genes.

MATERIALS AND METHODS

Materials. *E. coli* cpn60 and cpn10 and dimeric Rubisco from *R. rubrum* were purified and quantitated as in ref. 22. The concentrations of these proteins given in the text are

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Abbreviations: cpn60, chaperonin 60; cpn10, chaperonin 10; b-, mt-, and ch- (used as prefix), bacterial, mitochondrial, and chloroplast, respectively; Rubisco, ribulose-1,5-bisphosphate carboxylase.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M87646).

based on subunit molecular masses. The HPLC-TSK sizing column (type G3000SW, 7.5 × 600 mm) was from Pharmacia LKB.

Preparation of Rubisco-Depleted Chloroplast Extract. All steps were performed at 0°C. Intact chloroplasts, obtained from 10- to 12-day-old pea seedlings (26), were lysed by resuspension in 10 mM Hepes·KOH, pH 8/1 mM EDTA at a chlorophyll concentration of 1 mg/ml. The lysate was centrifuged at 47,000 × *g* (30 min) and the supernatant was recentrifuged at 150,000 × *g* (≈18 hr) to remove endogenous Rubisco. The Rubisco-free supernatant was concentrated to ≈20 mg of protein per ml (27) in a Centricon-10 (Amicon), adjusted to 50 mM Tris·HCl, pH 7.7/1 mM EDTA/5% (wt/vol) glycerol, and stored at −80°C.

Chaperonin-Assisted Refolding of Rubisco. The substrate for folding reactions was prokaryotic Rubisco, denatured in 10 mM HCl (28). To quantitate refolding, native Rubisco was treated identically, but KCl was substituted for HCl. Chaperonin-assisted refolding reactions (13) were conducted at 23°C to minimize spontaneous refolding (10). Reactions were terminated with glucose/hexokinase, and Rubisco activity was then determined (10, 13). Additional details appear in figure legends.

N-Terminal Sequence Analysis. N-terminal amino acid sequence analysis of the pea ch-cpn10 was carried out by automated Edman degradation, with a Porton PI2090E gas-phase sequencer, after electroblotting the protein from a 10–20% SDS/PAGE gradient gel, onto poly(vinylidine) difluoride (29). The gel samples used for electroblotting were similar to that shown in Fig. 2*B* (peak 1). The partial amino acid sequences for bovine liver mt-cpn10 (23) were obtained from the purified protein, after digestions with cyanogen bromide and trypsin. Detailed protocols are available upon request (to R.S.).

Isolation of a Spinach cpn10 cDNA Clone. A λgt11 spinach cDNA library (30) was screened using a digoxigenin-labeled DNA probe against chloroplast phosphoglycerate kinase. One positive clone, containing an additional cDNA insert, was subcloned in the vector Bluescript (Genofit, Geneva) and sequenced directly (31). The partial ch-cpn10 cDNA clone obtained in this manner was then used to generate a digoxigenin-labeled probe to screen a λgt10 spinach cDNA library for a full-length clone. Positive phage clones were subcloned as above, to yield the plasmid pSOCPN70/8.1. Both DNA strands were sequenced (31) using custom-designed primers.

Protein Import into Purified Chloroplasts. The ^{35}S-labeled spinach ch-cpn10 precursor was obtained by *in vitro* transcription-translation of pSOCPN70/8.1, in a rabbit reticulocyte lysate system (New England Nuclear), in the presence of [^{35}S]methionine (30). Protein import into isolated pea chloroplasts (equivalent to 30 μg of chlorophyll) was essentially as described (32).

RESULTS

Pea Chloroplasts Contain a Functional cpn10. The strategy for identifying ch-cpn10 was based on the premise that if such a protein were to exist, it might be functionally compatible with b-cpn60 (groEL). This approach was previously successful in identifying a mammalian mt-cpn10 (23). The starting material for ch-cpn10 was a pea chloroplast lysate, depleted of endogenous Rubisco. The soluble extract was applied to a gel filtration column (Fig. 1) and fractions were analyzed for their ability to assist b-cpn60 in the ATP-dependent, chaperonin-facilitated reconstitution of bacterial Rubisco. As previously documented (10, 13), under the conditions employed, chemically denatured Rubisco does *not* spontaneously refold to its native state but, instead, kinetically partitions to misfolded, aggregated structures. Active Rubisco can be recovered in high yield, however, when refolding reactions are conducted in the presence of b-cpn60, b-cpn10, and Mg·ATP (10, 13).

Column fractions (Fig. 1*A*) were therefore supplemented with b-cpn60 and nonnative Rubisco and incubated in the presence or absence of ATP. ATP-dependent Rubisco refolding activity eluted between 13.3 and 16.3 min, corresponding to an average molecular mass of ≈55 kDa. The active fractions were pooled and examined in greater detail (Fig. 1*B*). As anticipated, significant Rubisco refolding was only observed in the presence of b-cpn60, Mg·ATP, and

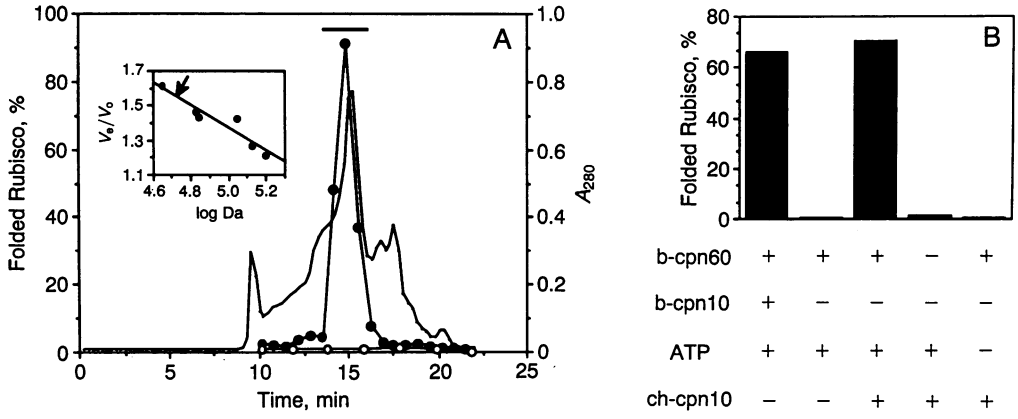

FIG. 1. A functional homolog of cpn10 in pea chloroplasts. (*A*) Rubisco-depleted chloroplast extract (4.4 mg of protein) was fractionated at 23°C on a TSK sizing column equilibrated with 100 mM Tris·HCl, pH 7.7/10 mM $MgCl_2$/10 mM KCl (buffer A). The flow rate was 1.0 ml/min and A_{280} was monitored (solid trace). Fractions (0.33 ml) were collected and assayed for Rubisco refolding. Acid-denatured Rubisco (10 μM) was rapidly diluted 100-fold into a 4°C solution of buffer A containing dithiothreitol (2 mM) and b-cpn60 (2.5 μM); subsequent steps were at 23°C. Column fraction aliquots (25 μl) were then incubated with 100 μl of this mixture and 75 μl of buffer A in the presence (●) or absence (○) of ATP (2 mM). After 1 hr, folding reactions were terminated and assayed for Rubisco activity (expressed as a percent of an equivalent amount of native Rubisco). (*Inset*) Molecular mass analysis of ch-cpn10, using aldolase (158 kDa), *R. rubrum* Rubisco dimer (112 kDa), bovine serum albumin monomer (67 kDa) and dimer (134 kDa), b-cpn10 (70 kDa), and ovalbumin (45 kDa) as standards. The arrow indicates "peak" folding activity at 14.8 min. V_e/V_o, elution volume/void volume. (*B*) The active fractions were pooled (horizontal bar in *A*) and concentrated to 12.2 mg of protein per ml. Rubisco-folding assays were conducted in 208 μl and contained 100 mM Tris·HCl (pH 7.7), 10 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, and 50 nM acid-denatured Rubisco. Some reactions (+ or −) also received b-cpn60 (1.25 μM), b-cpn10 (7.7 μM), ATP (2 mM), or pooled material from the TSK sizing column (48 μg of protein). Folding reactions were terminated after 30 min and assayed for Rubisco activity.

either b-cpn10 or the pooled fractions containing ch-cpn10. Thus, like its bacterial homolog groES, ch-cpn10 is essential as a cochaperonin and on its own does not facilitate Rubisco refolding. The successful reconstitution of Rubisco requires the joint participation of two distinct chaperonin components in a reaction in which ATP is utilized.

ch-cpn10 Forms a Stable Complex with b-cpn60. To identify the protein present in chloroplast extracts responsible for cpn10-like activity, we exploited the ability of b-cpn60 to form stable ATP-dependent complexes with cpn10s from homologous (9, 10) and heterologous (23) sources. Similar complexes also form in the presence of ADP (ref. 33 and unpublished results). Accordingly, the pooled material from the sizing column (Fig. 1*A*) was incubated with purified b-cpn60, in the absence or presence of ADP (Fig. 2). The reaction mixtures were then applied to the same sizing column and fractions corresponding to b-cpn60 (peak 1) and ch-cpn10 (peak 2) were analyzed by SDS/PAGE. As expected, when ADP was omitted (Fig. 2*A*), peak 1 contained *only* b-cpn60, whereas peak 2 contained a complex mixture of chloroplast proteins. However, when ADP (Fig. 2*B*) or ATP (not shown) was included, peak 1 contained b-cpn60 and an ≈24-kDa chloroplast protein that had previously eluted in peak 2. This result was unexpected since b-cpn10 and mt-cpn10 exhibit smaller molecular masses during SDS/PAGE: ≈15 kDa and ≈9 kDa, respectively (23).

Nevertheless, the isolated b-cpn60/ch-cpn10 complex could assist in the *in vitro* refolding of Rubisco, provided it was supplemented with ATP (Fig. 2*C*). In contrast, when identical experiments were performed with the material that eluted in peak 1 in the *absence* of ADP, no Rubisco refolding was observed. These results strongly implicate the ≈24-kDa chloroplast protein as a functional homolog of b-cpn10. However, more direct evidence for this notion was obtained when the putative ch-cpn10 was subjected to Edman degradation. As shown below, the N terminus of this protein exhibits striking homology to groES.

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ATVVAPKYTAIKPLGDRVLVK  ch-cpn10 (residues 1–21)

      MNIRPLHDRVIVK  groES (residues 1–13)
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Cloning of a ch-cpn10 from Spinach. During the course of screening a λgt11 cDNA library from spinach for phosphoglycerate kinase, a phage containing two cDNA inserts was isolated. One of these inserts showed obvious homology to b-cpn10 (below). A full-length cDNA clone (pSOCPN70/8.1) was subsequently isolated and further characterized. Its DNA and deduced primary amino acid sequences are shown in Fig. 3*A*. The open reading frame codes for a protein of a calculated molecular mass of 26,872 Da. Moreover, its N-terminal amino acid sequence exhibits typical characteristics of a chloroplast transit peptide (35), suggesting that this protein is synthesized in plant cytosol as a higher molecular mass precursor that is subsequently targeted to chloroplasts.

To test this possibility, the putative precursor was expressed *in vitro* in the presence of [^{35}S]methionine and used for chloroplast import studies. Upon incubation with intact chloroplasts the labeled spinach protein was taken up and processed to a smaller *mature* form of ≈24 kDa (Fig. 3*B*, asterisk). This protein and the Coomassie-stained pea ch-cpn10 (Fig. 2*B*) comigrate precisely during SDS/PAGE. A minor imported species (≈20% of the imported radioactivity) was also observed at ≈21 kDa (Fig. 3*B*). From the amino acid sequence of the cloned precursor protein (Fig. 3*A*) a transit peptide cleavage site could be deduced and lies between VRA and ASI. This processing site is in good agreement with the partial N-terminal sequence obtained from the purified pea ch-cpn10.

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ATVVAPKYTAIKPLGDRVLVK  pea (1–21)

VRA ASITTSKYTSVKPLGDRVLIK  spinach (51–74)
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Taken together, these observations argue strongly that the proteins that we have identified in pea and spinach chloroplasts—by two very different means—are one and the same. We conclude that the spinach ch-cpn10 is synthesized as a nuclear-encoded precursor with an N-terminal targeting sequence of ≈53 amino acid residues.

Two cpn10 Sequences Linked Together. The protomeric molecular mass of ch-cpn10 is about twice the size of b- or

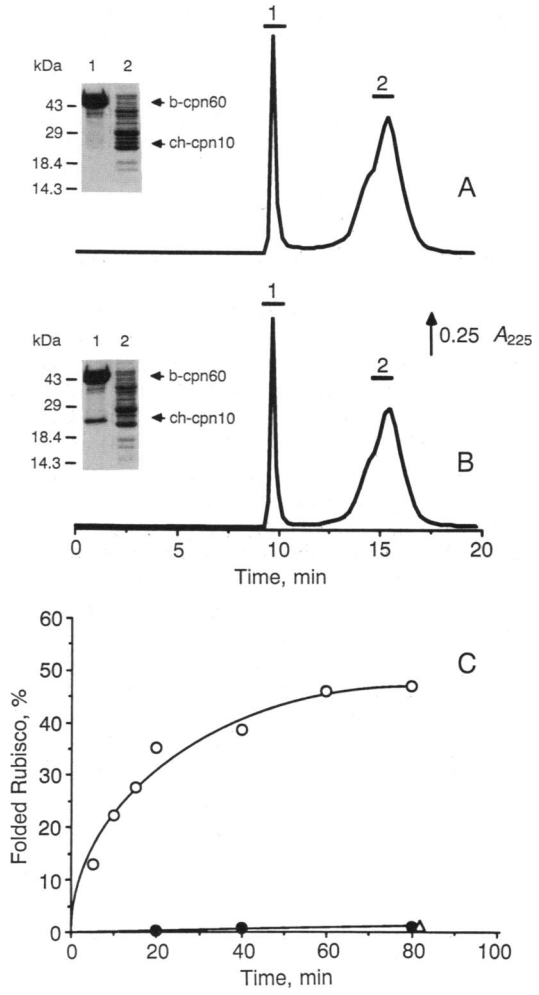

FIG. 2. Identification of pea ch-pn10: Formation of a stable complex with b-cpn60. (*A* and *B*) Reaction mixtures contained 88 mM Tris·HCl (pH 7.7), 8.8 mM $MgCl_2$, 8.8 mM KCl, 11.6 μM b-cpn60, and the pooled chloroplast proteins (≈2 mg/ml) from the TSK sizing column (Fig. 1*A*). The reaction mixture in *B* also contained ADP (0.65 mM). Following a 12-min incubation at 23°C, 200 μl was injected onto the same TSK sizing column used in Fig. 1. Proteins were eluted at 1 ml/min with buffer A (*A*) or with buffer A containing 0.25 mM ADP (*B*). Fractions corresponding to b-cpn60 (peak 1, 9.4–10.6 min) and ch-cpn10 folding activity (peak 2, 14.5–15.5 min) were collected and analyzed by SDS/PAGE (34) using 15% gels, after precipitating the proteins with 80% (vol/vol) acetone. (*C*) The fraction containing the isolated b-cpn60/ch-cpn10 complex (peak 1, *B*) was cooled to 4°C and supplemented with dithiothreitol (2 mM) and acid-denatured Rubisco to a concentration of 100 nM. The mixture was brought to 23°C and refolding was initiated by the addition of 2 mM ATP (○); the reaction shown in △ received no ATP. At given times, folding reactions were terminated and assayed for Rubisco activity. ●, Same experiment as in ○ using the material obtained from peak 1 (*A*).

FIG. 3. (*A*) Nucleotide sequence and deduced amino acid sequence of the spinach ch-cpn10 precursor. The coding region for the precursor protein starts at nucleotide 49. The sequence of the putative N-terminal chloroplast transit peptide is underlined. The first amino acid of *mature* ch-cpn10 (e.g., alanine; amino acid residue 54 of the precursor protein) starts at nucleotide 208. (*B*) Import of the spinach ch-cpn10 precursor into isolated chloroplasts. The radiolabeled ch-cpn10 precursor (lane 4) was incubated with isolated pea chloroplasts in the presence of 2 mM ATP. Following a 10-min import reaction, intact plastids were repurified by centrifugation through Percoll and either analyzed directly (lane 1) or treated with thermolysin (lane 2), to remove nonimported proteins. Analysis was by SDS/PAGE fluorography. Lane 3, molecular mass standards in kDa. The asterisk marks the position of the mature ≈24-kDa ch-cpn10.

mt-cpn10. This discrepancy is resolved in the sequence alignments shown in Fig. 4. Apparently, ch-cpn10 has arisen either through gene duplication or through the fusion of two distinct cpn10 genes. Thus, both halves of the *mature* spinach protein—the N-terminal portion (amino acids 9–104) and the C-terminal portion (amino acids 107–202)—exhibit remarkable homology to groES (9) and to bovine mt-cpn10 (23). Moreover, the two halves of the ch-cpn10 show 40% amino acid identity to each other and are ≈70% homologous if conservative changes are also considered. Although data will not be presented, a phylogenetic tree—based on 16 known prokaryotic cpn10 sequences—reveals that the N-terminal half of ch-cpn10 is more homologous to b-cpn10 from chlamydia (36), whereas the C-terminal half is more similar to b-cpn10 from cyanobacteria (37).

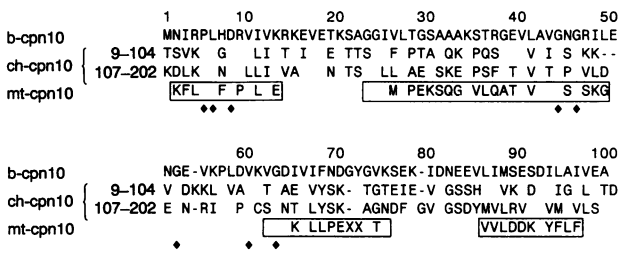

FIG. 4. The two halves of the mature spinach ch-cpn10 exhibit strong homology to b-cpn10 (groES). Two tandem amino acid sequences, present in the open reading frame of the spinach ch-cpn10 (amino acid residues 9–104 and 107–202 of the *mature* protein), and four partial sequences obtained from a purified bovine liver mt-cpn10 (22, 23) are aligned to the sequence of b-cpn10 (9). Blank spaces are residues identical to b-cpn10. Where indicated (–), single amino acid gaps have been introduced for best alignment. Boxes define the lengths of the mt-cpn10 partial sequences that were obtained by Edman degradation. Rhombs indicate the only eight residues that are identical in all b-cpn10 sequences present in the GenBank data base.

DISCUSSION

The role of cpn10 in chaperonin-assisted protein folding is not understood. It seems to function only as a cochaperonin—along with cpn60—and in certain cases is essential for the discharge of biologically active proteins from cpn60. Studies with Rubisco (10, 18) and rhodanese (12) suggest that the *in vitro* requirement for cpn10 depends primarily on whether a target protein can spontaneously refold to its native state under the prevailing conditions. ATP and even certain nonhydrolyzable analogs weaken the affinity of cpn60 for its nonnative protein substrates (12, 16–18). For proteins capable of spontaneous refolding, this alone may be sufficient to permit their partitioning to the native state. If spontaneous refolding is not possible, however, the mere release of a partially folded protein from cpn60 might result in its malfolding and/or aggregation (12, 18). In contrast, when cpn10 is also present, the protein species that are released from cpn60 are already apparently committed to the native state (12, 18). Whether this is accomplished through regulation of the cpn60 ATPase (9, 10, 12) remains to be determined.

The ability of cpn10 to form stable complexes with cpn60 in the presence of ATP (9, 10) was previously exploited to identify a mammalian mt-cpn10 (23) and, in the present study, a ch-cpn10 from higher plants. Indeed, these are the only nonprokaryotic cpn10 homologs reported. In both cases, the proteins identified by this approach are functionally compatible with b-cpn60 and exhibit significant sequence homology to b-cpn10. However, the plant protein is unique. It is nearly twice the expected size and is comprised of two complete prokaryotic-like cpn10 sequences, linked in tandem, in a

single protein. The conservation of identical amino acid residues between organisms as diverse as bacteria, plants, and mammals is likely to be important to cpn10 function. There are only eight such residues in the 16 bacterial cpn10 sequences that are known (Fig. 4). Remarkably, these same residues are present in *both* halves of the double ch-cpn10. Assuming that some selective pressure has operated in plants to maintain this situation, we speculate that both halves of the molecule might be able to function independently or perform different functions.

The presence of two distinct cpn60 subunits in chloroplasts (α and β) is also unique (2, 24, 25). That these two proteins are so divergent suggests that they have evolved to perform specialized functions (25), perhaps related to the assembly of higher plant Rubisco (21, 38). It is not known whether α and β reside within the same or different cpn60 tetradecamers. However, if chloroplasts do possess multiple isoforms of cpn60, the same might be true for its cochaperonin cpn10. In this regard, we cannot formally exclude the possibility that chloroplasts also contain a more conventional cpn10 homolog. In experiments similar to that shown in Fig. 2*B*, we have occasionally observed a faint band ($\approx$9 kDa) that also forms an ATP-dependent complex with b-cpn60. However, due to its low abundance, this protein has not yet been characterized. It is also conceivable that the double ch-cpn10 is actually cleaved into two prokaryotic-like cpn10 molecules, one or both of which is necessary for chloroplast function. However, there is currently no evidence for this notion, nor is it supported by *in vitro* chloroplast import data (not shown).

Several interesting structural questions remain to be answered regarding the physical interactions between ch-cpn10 and the 7-fold symmetrical b- and ch-cpn60. In our studies, the average "native" molecular mass of ch-cpn10 was $\approx$55 kDa, consistent with it being a dimer or a trimer. In contrast, groES (9) and other b-cpn10s are believed to be single heptameric toroids. However, gel filtration analysis might have underestimated its true molecular mass for various reasons, including (*i*) shape, (*ii*) nonspecific column interactions, and/or (*iii*) dissociation of the native oligomer under dilute *in vitro* conditions. Thus, it is too premature to predict the quarternary subunit composition of ch-cpn10.

In summary, we have identified and cloned a functional cpn10 homolog that is present in chloroplasts of higher plants. Clearly, the overexpression and purification of the *mature* recombinant spinach protein, as well as the two individual cpn10 motifs of which it is comprised, will help to clarify some of the important questions raised by this study. The availability of the purified recombinant protein should also prove useful in advancing our understanding of the role(s) chaperonin proteins play in the interrelated processes of chloroplast protein folding, assembly, and translocation.

Note. After the completion of this study, the amino acid sequence of a functional rat liver mt-cpn10 was published (39). Comparison with the partial sequences obtained from bovine mt-cpn10 (Fig. 4) reveals that these two proteins are highly homologous. It also appears that the C-terminal fragment shown for bovine mt-cpn10 (Fig. 4) should lie immediately adjacent to the preceding fragment to yield the following sequence: VGDKVLLPEXXGTKVVLDDKDYFLF.

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