Significance of the N-terminal Domain for the Function of Chloroplast cpn20 Chaperonin

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Chaperonins cpn60 and cpn10 are essential proteins involved in cellular protein folding. Plant chloroplasts contain a unique version of the cpn10 co-chaperonin, cpn20, which consists of two homologous cpn10-like domains (N-cpn20 and C-cpn20) that are connected by a short linker region. Although cpn20 seems to function like other single domain cpn10 oligomers, the structure and specific functions of the domains are not understood. We mutated amino acids in the “mobile loop” regions of N-cpn20, C-cpn20 or both: a highly conserved glycine, which was shown to be important for flexibility of the mobile loop, and a leucine residue shown to be involved in binding of co-chaperonin to chaperonin. The mutant proteins were purified and their oligomeric structure validated by gel filtration, native gel electrophoresis, and circular dichroism. Functional assays of protein refolding and inhibition of GroEL ATPase both showed (i) mutation of the conserved glycine reduced the activity of cpn20, whether in N-cpn20 (G32A) or C-cpn20 (G130A). The same mutation in the bacterial cpn10 (GroES G24A) had no effect on activity. (ii) Mutations in the highly conserved leucine of N-cpn20 (L35A) and in the corresponding L27A of GroES resulted in inactive protein. (iii) In contrast, mutant L133A, in which the conserved leucine of C-cpn20 was altered, retained 55% activity. We conclude that the structure of cpn20 is much more sensitive to alterations in the mobile loop than is the structure of GroES. Moreover, only N-cpn20 is necessary for activity of cpn20. However, full and efficient functioning requires both domains.

It is now well accepted that protein folding in the cell requires the assistance of a family of proteins known as chaperones (1). One family of chaperones, known as Type I “chaperonins,” is composed of two members that cooperate to fold proteins in the cell during processes of synthesis, translocation, or stress (2). The prototypes of these proteins are the bacterial GroEL (chaperonin 60 or cpn60) and GroES (chaperonin 10 or cpn10). GroEL is a cylindrical oligomer constructed of two back-to-back heptameric rings, of 60 kDa subunits each. The co-chaperonin GroES is a smaller ring formed from seven identical 10-kDa subunits. Unfolded proteins bind to the inner cavity of one ring of the GroEL cylinder, and are released by the binding of ATP and GroES to the opposing toroid. The structure and mechanism of these proteins from Escherichia coli have been extensively reviewed (3, 4).

A similar system was discovered in chloroplasts as a factor assisting in Rubisco assembly (5). Early studies indicated that the chloroplast proteins were structurally distinct from GroEL and GroES (for a review see Ref. 6). Whereas the bacterial and mammalian cells contain only one homolog each of cpn60 and cpn10, the plant cell codes for several homologs of the chaperonin proteins. A survey of the Arabidopsis thaliana genome has identified 29 genes that potentially encode chaperonins (7). Of these, 9 contain chloroplast signal sequences, three of them for co-chaperonin homologs. One of these homologs was previously identified in both pea and spinach as being twice the size of the classic cpn10 co-chaperonin (8). Sequence analysis showed that the protein was composed of two linked cpn10 homologs, joined by a putative linker region of Thr-Asp-Asp-Val-Lys-Asp (9). This double co-chaperonin, called cpn20 (ch-cpn20 or cpn21), contained a signal sequence and was shown to be imported into the chloroplast. In vitro, it was capable of assisting GroEL and chloroplast cpn60 in refolding denatured Rubisco (8, 10, 11). Cross-linking studies indicated that spinach cpn20 existed as many oligomeric species in solution, however, the conformation bound by GroEL has yet to be elucidated (12).

Cloning of the homologous gene from A. thaliana showed it to be 61% identical to that from spinach (13, 14). Structural studies suggested that, in contrast to the heptameric GroES prototype, this homolog was tetrameric: both native gel electrophoresis and gel filtration experiments gave an estimated molecular mass of 70 – 80 kDa (13, 14). Tobacco plants transformed with heterologous cpn20 under a constitutive promoter accumulated the protein in chloroplast stroma, confirming chloroplast localization. In line with its function as a heat shock protein, Northern blot analysis showed that the Arabidopsis cpn20 was heat-inducible (14).

The two cpn10-like domains of Arabidopsis cpn20 are 46% identical to each other (13, 14). Each side contains the highly conserved N-terminal PLXDRV motif and has its own “mobile loop,” the region that was shown to be responsible for binding
to cpn60 (15, 16). Two studies published a decade ago on the spinach homolog of cpn20 cloned each domain separately to determine the role of each. Both studies found that the individual domains were not functional in vitro, but did exhibit some activity in vivo, suggesting that each of the two domains may be able to function independently (9, 17). However, the lack of activity in vitro may suggest that overall oligomeric structure is necessary for full function. Thus, the significance of the double domain structure remains unclear.

In this work, we mutated conserved amino acids in the mobile loops of A. thaliana cpn20. Our approach used information from previous mutation analyses of GroES, which were able to map amino acids critical for binding to GroEL. We altered conserved mobile loop amino acids in the N-terminal cpn10-like domain (N-cpn20), the C-terminal cpn10-like domain (C-cpn20), or in both. Structural analyses were carried out on the purified proteins to ensure their oligomeric integrity and their functionality was tested in vitro. Overall, our results suggest that the structure of cpn20 is much more sensitive to changes in the mobile loop region than it is GroES. We also show that whereas the primary protein folding assistance by cpn20 resides in its N-terminal domain, maximal activity of the protein requires a functional C terminus as well.

**Experimental Procedures**

Preparation of Single Domain (truncated) cpn20 Constructs—

The N-cpn20 and C-cpn20 homologs of mature wild type cpn20 from A. thaliana (At5g20720) were cloned individually by PCR into pGEM T-easy (Promega) with primers that added the restriction site NdeI before the N terminus of each domain and Xhol after the C terminus of each domain, as follows: for N-cpn20: cpn20-1, 5'-TAACATATGCTTGTTGGTCG-CCCTAAG-3'; cpn20-2, 5'-TAACCTGAGCTGTGTCTCAAGAATGCC-3'; for C-cpn20: cpn20-3, 5'-TAACATATAAGGATCTCCAACCTTGAAAT-3'; cpn20-4, 5'-TAACCTGAGGAGAAATGAGCCATACCATC-3'. The constructs were further cloned into pET22b+ (Novagen), and overexpressed with a His6 tag derived from the vector.

Preparation of cpn20 Mutants—

Mutagenesis was carried out on pGEM-T-easy (Promega) containing the mature wild type A. thaliana cpn20 (At5g20720) gene, using a QuikChange site-directed mutagenesis kit (Stratagene). The following primers were used to introduce site-specific mutations (obtained from Yechezkel Kashi at the Technion Institute of Technology, Haifa, Israel) were diluted 1:10 into the same medium and grown at 37 °C to an A600 of 0.7. Overexpression was then induced for 3 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside at the same temperature. Cells were harvested by centrifugation for 10 min at 6000 × g in a SLC-4000 rotor. For the preparation of cell-free extracts, cell pellets were resuspended 1:7 (w/v) in 20 mM Na-HEPES, pH 7.4, 5% glycerol, 200 mM NaCl, 30 mM imidazole, 1 μg/ml protease inhibitor mixture (pepsatin, chymostatin, antipain, leupeptin, aprotinin, all purchased from Sigma), 0.5 mM phenylmethylsulfonyl fluoride, and 1,500 units DNase (Sigma), homogenized, and passed through a microfluidizer. Debris was removed by centrifugation for 30 min at 35,000 × g in an ultracentrifuge in a Ti-70 rotor. Supernatant was bound to nickel-agarose resin (Rimon Biotech), washed, and eluted with 300 mM imidazole. Relevant fractions were concentrated and desalted (PD10 columns, GE Healthcare) to a final buffer of 20 mM Na-HEPES, pH 7.4, 200 mM NaCl, and 5% glycerol. Single domain proteins were further purified on a Superdex (GE Healthcare) 200 gel filtration column in 20 mM Na-HEPES and 300 mM NaCl.

Purification of GroES and GroES Mutants—

GroES constructs described above were overexpressed in E. coli BL21(DE3). Overnight cultures in Luria broth containing ampicillin (100 μg/ml) were diluted 1:10 into the same medium and grown at 37 °C to an A600 of 0.7. Overexpression was then induced for 3 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside at the same temperature. Cells were harvested by centrifugation for 10 min at 6000 × g in a SLC-4000 rotor. For the preparation of cell-free extracts, cell pellets were resuspended 1:7 (w/v) in 20 mM Na-HEPES, pH 7.4, 5% glycerol, 200 mM NaCl, 30 mM imidazole, 1 μg/ml protease inhibitor mixture (pepsatin, chymostatin, antipain, leupeptin, aprotinin, all purchased from Sigma), 0.5 mM phenylmethylsulfonyl fluoride, and 1,500 units DNase (Sigma), homogenized, and passed through a microfluidizer. Debris was removed by centrifugation for 30 min at 35,000 × g in an ultracentrifuge in a Ti-70 rotor. Supernatant was bound to nickel-agarose resin (Rimon Biotech), washed, and eluted with 300 mM imidazole. Relevant fractions were concentrated and desalted (PD10 columns, GE Healthcare) to a final buffer of 20 mM Na-HEPES, pH 7.4, 200 mM NaCl, and 5% glycerol. Single domain proteins were further purified on a Superdex (GE Healthcare) 200 gel filtration column in 20 mM Na-HEPES and 300 mM NaCl.

Preparation of GroES Mutants—

GroES in pTRC99a (obtained from Yechezkel Kashi at the Technion Institute of Technology, Haifa, Israel) served as a template for mutagenesis, using the QuikChange site-directed mutagenesis kit (Stratagene). The following primers were used to introduce site-specific mutations: G24A1, 5'-GAGAACTAAATCGTGTGCGCAAATCGTCTTGACCGGC-3'; G24A2, 5'-AGCCGGTCAAGACATTGGCCGCAGACATTGATTC-3'; G24A1, 5'-GCTGCGGCGCACTGTTGACCGGC-3'; L27A1, 5'-GCTGCGGCGCACTGTTGACCGGC-3'; L27A2, 5'-GCTGCGGCGCACTGTTGACCGGC-3'. All constructs were fully sequenced to confirm the presence of the desired mutation.

Purification of Ch-cpn20 Mutants, Single Domain (Truncated) Proteins, and Wild Type Proteins—

Ch-cpn20 constructs described above were overexpressed in E. coli BL21(DE3). Overnight cultures in Luria broth containing ampicillin (100 μg/ml) were diluted 1:10 into the same medium and grown at 37 °C to an A600 of 0.7. Overexpression was then induced for 3 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside at the same temperature. Cells were harvested by centrifugation for 10 min at 6000 × g in a SLC-4000 rotor. For the preparation of cell-free extracts, cell pellets were resuspended 1:7 (w/v) in 20 mM Na-HEPES, pH 7.4, 5% glycerol, 200 mM NaCl, 30 mM imidazole, 1 μg/ml protease inhibitor mixture (pepsatin, chymostatin, antipain, leupeptin, aprotinin, all purchased from Sigma), 0.5 mM phenylmethylsulfonyl fluoride, and 1,500 units DNase (Sigma), homogenized, and passed through a microfluidizer. Debris was removed by centrifugation for 30 min at 35,000 × g in an ultracentrifuge in a Ti-70 rotor. Supernatant was bound to nickel-agarose resin (Rimon Biotech), washed, and eluted with 300 mM imidazole. Relevant fractions were concentrated and desalted (PD10 columns, GE Healthcare) to a final buffer of 20 mM Na-HEPES, pH 7.4, 200 mM NaCl, and 5% glycerol. Single domain proteins were further purified on a Superdex (GE Healthcare) 200 gel filtration column in 20 mM Na-HEPES and 300 mM NaCl.
A

Arab: SKYAGTEVEFNDVHLKILKEKDDLVIDGEDTEDIKELKFLNDRVFIVERKVAEZEK
Spin: SKYTGTITGDVSGLLHRLKDGDIEDVDKELKFLNRLLLKVAEVENK

N-terminal Domain of Chloroplast Chaperonin 20

B

| Protein          | GroES.E.coli | N-cpn20.A. thaliana | N-cpn20. Spinach | C-cpn20.A. thaliana | C-cpn20. Spinach |
|------------------|--------------|---------------------|------------------|---------------------|------------------|
| **Top**          |              |                     |                  |                     |                  |
| GroES.E.coli     | EVETKSAGGIVLTGSA |                     |                  |                     |                  |
| N-cpn20.A. thaliana | EAEKTLGGLLPSTAA |                     |                  |                     |                  |
| N-cpn20. Spinach | IVEKTT9GFLPSTAA |                     |                  |                     |                  |
| C-cpn20.A. thaliana | EAEKTLGGLLPSTAA |                     |                  |                     |                  |
| C-cpn20. Spinach | EVENKSYGSLLAESSK |                     |                  |                     |                  |
| Cpn10. Mouse     | AAE2VTKGIMLFEKQQ |                     |                  |                     |                  |
| Cpn10. Yeast     | KAAKTAAGLHYLFKENV |                     |                  |                     |                  |
| Ch-cpn10.A. thaliana | DLP1FSSGVIKNFLPAV |                  |                  |                     |                  |
| Gp31.BPT4        | GDEEVTE9G3L1LKRQV |                     |                  |                     |                  |

FIGURE 1. A, linker region between the two cpn10-like domains of cpn20. An alignment of the area surrounding the linker region between the two cpn10-like domains of cpn20 in A. thaliana (Arab) and Spinacea oleracea (Spin) is presented. The putative linker region is marked by a gray box. Division sites chosen in previous studies (bottom) and this study (top) are indicated by arrows. B, comparison of the mobile loop from cpn10 homologs. Sequence alignment of the mobile loop region of cpn10 homologs from various sources is presented. Amino acids that were chosen for mutagenesis in this study are indicated with an asterisk.

gradient of NaCl in 20 mM Na-HEPES, pH 7.4, at a flow rate of 1 ml/min. Fractions (3 ml) enriched with GroES were eluted between 200 and 300 mM NaCl. These were pooled, concentrated, and further purified by gel filtration on a Superdex 200 column (Amersham Biosciences), pre-equilibrated with 20 mM Na-HEPES and 200 mM NaCl, and eluted at a flow rate of 1 ml/min. Fractions (3 ml) enriched for GroES were concentrated and stored in aliquots at −80 °C.

GroEL Purification—GroEL in pTRC99a (obtained from Yechezkel Kashi at the Technion Institute of Technology, Haifa, Israel) was overexpressed in E. coli DH5α cells and purified using two ion-exchange dimensions followed by gel filtration, similar to previously described methods (19).

Protein Determination—Protein concentration was determined using the bicinchoninic acid protein assay kit (Sigma) using bovine serum albumin as a standard. Protein concentrations refer to monomer concentration unless otherwise indicated.

In Vitro Refolding of Heat-denatured Malate Dehydrogenase (MDH)—Thermal denaturation of mitochondrial MDH (0.33 mM) was performed at 47 °C for 30 min in the presence of 10 μM GroEL, 50 mM Na-HEPES, pH 7.4, 50 mM KCl, 10 mM MgCl2, and 5 mM dithiothreitol. The chaperonin-assisted MDH refolding was initiated by bringing the sample to room temperature and adding co-chaperonin at various concentrations and 2 mM ATP (20). Following a 1-h incubation either at room temperature or 37 °C, aliquots were removed and assayed for MDH activity by adding a reaction mixture containing 150 mM potassium phosphate, pH 7.6, 10 mM dithiothreitol, 0.28 mM NADH, and 0.5 mM oxaloacetate. MDH activity was determined by monitoring the oxidation of NADH as a function of time at 340 nm (21). Refolding yields are expressed as a percentage of maximal refolding obtained using GroES. Spontaneous refolding of MDH was generally 1–2%, whereas in the presence of ATP alone, refolding yields were 5–10%. The refolding reaction using GroES generally yields about 80% of an equal amount of native MDH.

ATP Hydrolysis—The ATPase assay used in this study couples the conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase and the conversion of pyruvate to lactate by lactate dehydrogenase. During this process, the oxidation of NADH is monitored at 340 nm. Each 1-ml reaction mixture contained: GroEL (5 μM), 10 μM co-chaperonin, 50 mM Na-HEPES, pH 7.4, 50 mM KCl, 10 mM MgCl2, 0.3 mM NADH, 0.2 mM phosphoenolpyruvate, 20 units of pyruvate kinase, and 10 units of lactate dehydrogenase. The reaction was initiated by the addition of 2 mM ATP. A reduction in the absorbance of NADH was monitored at 340 nm for 4 min. Rates were extracted from the linear phase of the reaction.

Native Gels—Proteins (10 μg) were separated on 10% polyacrylamide mini-gels using the Laemmli buffer system and omitting SDS and β-mercaptoethanol (22).

Circular Dichroism Spectroscopy (CD)—CD experiments were conducted using an Aviv CD spectrometer model 202. Wavelength scans were performed using 1 mg/ml of protein diluted in 5 mM Na-HEPES, pH 7.4, and 50 mM NaCl, in a 0.1-mm cell at increasing temperatures. Each spectrum was obtained by averaging 5 spectra recorded from 260 to 190 nm. Data were recorded every 1 nm with an averaging time of 4 s. The protein was equilibrated for 1 min at each temperature before initiating the scan. Raw data were corrected for buffer contribution, smoothed, and converted to molar ellipticity.

CD temperature scans were performed in a 1-mm path cuvette at a protein concentration of 0.3 mg/ml. Scans were performed by varying the temperature from 4 to 80 °C and data were collected every 1 °C with an equilibration time of 30 s. Ellipticity was monitored at 222 nm.

RESULTS AND DISCUSSION

Mutagenesis Strategy—The double cpn20 constitutes an intriguing divergence from classic GroES-like cpn10 molecules in that it is composed of two GroES-like domains. The first logical experiment in attempting to explain the significance of such redundancy would be to separate the homologous domains. Single domain clones from spinach chloroplast were previously designed to address this topic and found inactive in vitro (9, 17). In vivo, some activity was reported for the individual domains. Upon close examination, we found that the division site chosen between the two cpn10-like domains did not fall in the purported linker site (9) (Fig. 1A); instead, it fell within the C terminus of the N-domain. Suspecting that this might explain the observed in vitro inactivity of the protein
domains, we began by separately cloning each cpn10-like domain of cpn20 from *A. thaliana*, dividing the gene in the center of the purported linker region (Fig. 1A). As in the previously published works, these halves were inactive *in vitro* (data not shown).

The results may suggest that the whole molecular configuration and perhaps inter-domain interaction is required for stability and function of the protein. Thus, we utilized a different approach to analyzing the contribution of each domain for function of the protein, which preserved the larger order molecular interactions. This approach involved mutating amino acids critical for binding to GroEL, in one domain or the other, thereby allowing us to inactivate each domain separately.

The amino acids chosen for mutagenesis were based on extensive structural studies of the bacterial GroES, which have identified residues important for GroEL binding. The unstructured mobile loop region of GroES was first identified as being important for the binding of co-chaperonin to GroEL (15, 16). This mobile loop is highly conserved in all known cpn10 homologs (23). Further mutagenesis and other studies confirmed that the “IVL” motif within the GroES mobile loop was primarily responsible for the affinity of cpn10 to cpn60 (15, 16, 23, 24), whereas the preceding GG allowed for formation of the hairpin that is adopted upon binding to GroEL (15, 16). Other amino acids in the mobile loop region were also shown to contribute to the affinity of cpn10 to cpn60 (23).

An alignment of the mobile loop region from the N-cpn20, C-cpn20, and cpn10 from various origins is presented in Fig. 1B. The mutations carried out in the present study are labeled with an asterisk. In one set of mutants, one of the conserved Gly was mutated to Ala. In the other set of mutants, the highly conserved L of the GroES “IVL” binding site (ILL in N-cpn20 and LL in C-cpn20) was mutated to Ala. The mutations were carried out in the N-terminal domain (G32A,L35A), the C-terminal domain (G130A,L133A), or in both cpn10-like domains of the full-length cpn20 (GDM-glycine double mutant; LDM-leucine double mutant). For comparison, the same mutations were made in GroES (G24A,L27A).

**Purification and Structural Properties of Wild Type and Mutated Ch-cpn20**—Wild type and mutant cpn20 were purified as His-tagged proteins on nickel-agarose resin. GroES and its mutants were purified as described under “Experimental Procedures.” A gel showing purified mutants is shown in Fig. 2. The integrity of the oligomeric structure and stability of the purified mutants were examined using gel filtration, native gel electrophoresis, and CD spectroscopy. Gel filtration analysis showed that all the purified mutants, wild type cpn20, and GroES eluted primarily in 2–3 fractions when run on a Superdex 200 gel filtration column. Although the GDM mutant elutes in the same fractions, it appears over a broader range of higher oligomeric species than the others (Fig. 3). These results are supported by native gel electrophoresis showing that cpn20 and most of its mutated forms migrate slightly more slowly than GroES, whose oligomeric molecular mass is 70 kDa (data not shown). This is consistent with reports published previously suggesting that ch-cpn20 from *A. thaliana* forms a tetrameric structure (13, 14). Similar to results from the gel filtration, native gel electrophoresis showed that the GDM double mutant seemed to form a significant amount of higher order structures, indicating that mutation of the conserved glycines on both domains has a deleterious effect on the structure of cpn20.

Further support for a native-like structure was obtained from CD spectra of the proteins between 260 and 190 nm at various temperatures. Fig. 4, A–C, present sample traces of representative cpn20 mutants L35A and G130A compared with wild type ch-cpn20. Interestingly, the double cpn20 exhibits a similar CD spectrum to GroES, indicating a similar secondary structure. Moreover, for all the mutants, a clear transition between 200 and 250 nm was observed at temperatures greater than 60 °C, similar to the results previously reported for GroES (25). In addition to the spectra, thermal denaturation was monitored at 222 nm between 4 and 80 °C and indicated a similar degree of thermal stability for cpn20 and all the mutant proteins, with a *Tm* of ~50 °C (representative curves in Fig. 4D; all cpn20 mutants in supplemental data). Despite a similar *Tm*, the denaturation curve of GDM was biphasic (Fig. 4E). The increase in ellipticity at higher temperatures observed for GDM indicates that the protein aggregated (26) and this was supported by a large amount of white precipitate in the cuvette at the end of the experiment. Overall, the results indicate that, with the excep-
tion of the GDM double mutant, the mutations did not affect the oligomeric structure of the proteins.

Functionality of the cpn20 and GroES Mutants—The purified mutants and wild type cpn20 were tested for their ability to assist GroEL in mediating protein refolding of denatured MDH at 25 °C. A summary of refolding results can be seen in Table 1. The G32A and G130A mutants were able to assist GroEL in the refolding of denatured MDH with maximum yields of 80 and 60%, respectively, relative to those obtained with GroES and wild type ch-cpn20 (Fig. 5; Table 1). It should be noted that for all refolding reactions, identical results were obtained when chloroplast cpn60 was used instead of GroEL (supplemental data). In addition to the lower yield, the monomer ratio of co-chaperonin: chaperonin that gives 1⁄2 the plateau level of refolding (EC_{50}) for the Gly mutants is about 1. This is approximately four times greater than that observed for GroES and cpn20, indicating a lower relative affinity of the Gly mutants for GroEL (Table 1). Consistent with these results, G32A and G130A were able to inhibit the ATPase activity of GroEL, but not to the same extent as GroES or wild type cpn20 (Fig. 6).

Previous studies highlighted the significance of the “GG” sequence for co-chaperonin function. It was shown that the glycine residues are needed to ensure flexibility required for the mobile loop β hairpin conformation (15, 16). However, bacteria containing GroES with mutations in Gly-24 were viable, suggesting that perhaps one glycine at this position is sufficient for function of GroES (16, 27, 28). Consistent with these reports, the GroES mutant G24A was equally as active as wild type GroES at room temperature with a similar EC_{50}, and was able to inhibit GroEL ATPase (Table 1; Fig. 6). Interestingly, it exhibited only an 80% refolding yield at 37 °C. In contrast, a mutation in the single glycine of the homologous Gp31, which contains only one glycine residue at this position, abolished protein function and resulted in a lethal phenotype for bacteriophage T4 growth (24).

Because N-cpn20 and C-cpn20 both have a double Gly, we did not expect to find that the G-A mutants would be significantly affected in their ability to assist GroEL in mediating protein folding at 25 °C (60 and 80% yield for the single mutants compared with 100% with the same GroES mutant). Thus, it seems as though the oligomeric structure of cpn20 is more sensitive in changes in the mobile loop, and both glycines of the doublet are required for full activity. Two possible reasons, which are not mutually exclusive, can be used to explain the lower activity of these Gly mutants. First, due to extra spatial constraints dictated by the unique double domain structure of cpn20, it is possible that even a single glycine mutation could alter the orientation of the mobile loop, hence preventing it from proper interaction with GroEL. Second, these results may also indicate that efficient function of cpn20 requires both domains of the molecule.

Even more surprising were results obtained with the GDM, which had mutations in both Gly-32 and Gly-130. This con-

FIGURE 4. Circular dichroism analysis of cpn20 mutants. A circular dichroism spectrum of cpn20 (A), cpn20 mutant L35A (B), and cpn20 mutant G130A (C) was performed at various temperatures. The represented spectra were obtained by averaging 5 spectra, correcting for buffer contribution and smoothing. Molar ellipticity as a function of wavelength is presented for each protein. Thermal denaturation curves were carried out for cpn20 wild type and mutants L35A and G130A (D) and cpn20 mutant GDM (E) by varying the temperature from 4 to 80 °C. Data were collected every 1 °C with an equilibration time of 30 s. Ellipticity was monitored at 222 nm.
The second mutation investigated was engineered within the IVL motif, which was shown to be directly responsible for binding of co-chaperonin to chaperonin (15, 16). Mutant L133A, in the C-terminal domain of cpn20, was able to refold about 55% of the denatured MDH and exhibited an $EC_{50}$ of about 1.5, whereas L35A, containing the same mutation in the N-terminal domain, was not able to assist GroEL in refolding MDH at all (Fig. 5). Likewise, the L double mutant, in Leu-35 and Leu-133, was not active. The ability of these mutants to inhibit GroEL ATPase was consistent with their capacity to assist GroEL at protein folding (Fig. 6). By way of comparison, the same mutation in GroES (L27A) showed no refolding activity and did not inhibit GroEL ATPase (Table 1, Fig. 6).

These results indicate that at 25 °C, it is sufficient to have a functional N-cpn20 for cpn20 to be active. However, the plateau level of refolding yield is still significantly lower than that of the wild type molecule, implying that function of the C-cpn20 is required for more efficient activity. Because L35A is completely inactive, and the GroES tripeptide was shown to be responsible for binding to cpn60, our results may also suggest that binding of the C-cpn20 requires proper binding of N-cpn20 as a prerequisite. Indeed, direct binding experiments carried out using gel filtration indicated that all active mutants bind GroEL, whereas inactive mutants did not (supplemental data).

Except for G130A, none of the cpn20 mutants in this study was able to support the refolding of denatured MDH when the reaction was carried out at 37 °C. Given that mutants exhibiting refolding activity expressed a significantly lower apparent affinity for GroEL than GroES, this result is not surprising. It was previously shown that the affinity of GroES for GroEL decreases with increasing temperature (20). Thus, the results are consistent with a temperature-dependent decrease in affinity that apparently precludes binding of the mutants to GroEL. The only mutant to show any activity at 37 °C was the G130A mutant (~30% yield; Table 1). Although we are not fully able to explain this phenomenon, it should be noted that again, the mutation here is in the C terminus, whereas the same mutation in the N-domain was inactive at this temperature. This further supports our theory that a functional N terminus is necessary for activity of the molecule.

**Conclusion**—In this study, we characterized the effect of two different mutations in the mobile loop region of cpn20. The first is the universally conserved glycine, which was suggested to be responsible for the flexibility of the mobile loop. The second, leucine, is part of the IVL tripeptide of GroES that was shown to be directly involved in the binding of the co-chaperonin to GroEL. Mutation of one of the glycines in the mobile loop of either N-cpn20 (G32A) or C-cpn20 (G130A) causes a reduction in the ability of the molecule to function at room temperature. Because the same mutation in GroES (G24A) has no effect, we can deduce that the mobile loop region of cpn20 is much more sensitive to alterations than is GroES. This can be attributed to the complex and unique oligomeric structure of cpn20. In addition, the method of characterization we undertook enabled us to neutralize each domain separately, thus enabling us to explore the double domain structure. The fact that mutant L35A is inactive, yet the corresponding L133A is active, suggests that the two domains are not uniform in their structural requirements.
suggests that a functional N-terminal domain is necessary and sufficient for basic activity of the cpn20 molecule. However, the fact that mutant L133A is only partially active at 25 °C and inactive at 37 °C suggests that full and efficient activity of the molecule is dependent on contributions from both cpn10-like domains.

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