Differential effects of co-chaperonin homologs on cpn60 oligomers

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Abstract In this study, we have investigated the relationship between chaperonin/co-chaperonin binding, ATP hydrolysis, and protein refolding in heterologous chaperonin systems from bacteria, chloroplast, and mitochondria. We characterized two types of chloroplast cpn60 oligomers, ch-cpn60 composed of α and β subunits ($\alpha_7\beta_7$ ch-cpn60) and one composed of all β subunits (β_{14} ch-cpn60). In terms of ATPase activity, the rate of ATP hydrolysis increased with protein concentration up to 60 μ M, reflecting a concentration at which the oligomers are stable. At high concentrations of cpn60, all cpn10 homologs inhibited ATPase activity of $\alpha_7\beta_7$ ch-cpn60. In contrast, ATPase of β_{14} ch-cpn60 was inhibited only by mitochondrial cpn10, supporting previous reports showing that β_{14} is functional only with mitochondrial cpn10 and not with other cpn10 homologs. Surprisingly, direct binding assays showed that both ch-cpn60 oligomer types bind to bacterial, mitochondrial, and chloroplast cpn10 homologs with an equal apparent affinity. Moreover, mitochondrial cpn60 binds chloroplast cpn20 with which it is not able to refold denatured proteins. Protein refolding experiments showed that in such instances, the bound protein is released in a conformation that is not able to refold. The presence of

glycerol, or subsequent addition of mitochondrial cpn10, allows us to recover enzymatic activity of the substrate protein. Thus, in our systems, the formation of co-chaperonin/chaperonin complexes does not necessarily lead to protein folding. By using heterologous oligomer systems, we are able to separate the functions of binding and refolding in order to better understand the chaperonin mechanism.

Keywords Chaperonin · GroEL · GroES · Chloroplast · Mitochondria · cpn20 · cpn60 · cpn10 · Protein folding · Co-chaperonin

Abbreviations

ch-cpn60	type I chloroplast 60-kDa chaperonin
$\alpha_7\beta_7$ ch-cpn60	type I chloroplast 60-kDa chaperonin that was reconstituted in vitro from α and β subunits
β_{14} ch-cpn60	type I chloroplast 60-kDa chaperonin that was reconstituted in vitro from only β subunits
ch-cpn20	plant chloroplast cpn20
mt-cpn60	mammalian mitochondrial cpn60
mt-cpn10	mammalian mitochondrial cpn10
mMDH	mitochondrial malate dehydrogenase
DTT	dithiothreitol
ATP	adenosine triphosphate
Rubisco	ribulose 1,5 biphosphate carboxylase

Introduction

Chaperonins are a family of homologous oligomeric proteins that mediate the folding of newly translated, newly translocated, and stress-denatured proteins (Hartl 1996; Horwich et al 2007). Those of type I function as a team

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of two proteins: the 60-kDa chaperonin and its 10-kDa co-chaperonin. They are found in bacteria as well as in eukaryotic cellular compartments thought to be of endosymbiotic origin. The chloroplast chaperonin, ch-cpn60, was originally discovered as a high-molecular-weight complex that was non-covalently bound to ribulose 1,5 biphosphate carboxylase (Rubisco) large subunits pending their incorporation into holoenzyme, in chloroplasts (reviewed in Ellis and van der Vies 1991; Gatenby and Ellis 1990). Subsequent studies have implicated ch-cpn60 not only in the folding of Rubisco (Hemmingsen et al 1988) but also of a large number of chloroplast proteins (Bonk et al. 1996, 1997; Kessler and Blobel 1996; Lubben et al. 1989; Molik et al. 2001; Tsugeki and Nishimura 1993).

Similar to the well-studied bacterial GroEL oligomer, ch-cpn60 is a cylindrical tetradecamer composed of two seven-member rings capable of refolding denatured proteins in vitro in the presence of adenosine triphosphate (ATP) and homologs of the 10-kDa co-chaperonins (cpn10; for a review, see Boston et al. 1996). In contrast to the 14 identical subunits of GroEL, ch-cpn60 oligomers were shown to be composed of two divergent subunit types, α and β (Bonk et al. 1996; Martel et al. 1990), which reside in the same oligomer (Nishio et al. 1999). Moreover, analysis of the *Arabidopsis* genome revealed multiple isotypes of both α and β subunits (Hill and Hemmingsen 2001).

The co-chaperonins from bacteria and mitochondria are both heptameric rings of 10-kDa subunits (Hunt et al. 1996; Viitanen et al. 1998). In contrast, the first chloroplast cpn10 gene to be isolated consists of two cpn10-like sequences fused head-to-tail and is known as cpn20 (Bertsch et al. 1992) or cpn21 (Hirohashi et al. 1999). While the oligomeric structure of the chloroplast cpn20 (ch-cpn20) protein has not been unequivocally determined (Koumoto et al. 1999; Sharkia et al. 2003), the protein seems to function similarly to GroES in terms of its ability to assist cpn60 homologs in their protein-folding tasks in vitro (Dickson et al. 2000; Viitanen et al. 1995). Subsequently, a homologous single-domain chloroplast cpn10 was cloned from *Arabidopsis thaliana* and characterized in vitro (Koumoto et al. 2001; Sharkia et al. 2003). The reason for multiple homologs in the same cellular compartment remains obscure; however, possible differences in subcellular localization have been suggested in the past (Schlicher and Soll 1996).

Ch-cpn60 purified as an oligomer from pea chloroplast was shown to refold Rubisco from *Rhodospirillum rubrum* and mammalian mitochondrial malate dehydrogenase (mMDH; Viitanen et al. 1995). Similar to GroEL, the refolding was equally as efficient whether assisted by co-chaperonins from chloroplast, mitochondrial, or bacterial sources (Viitanen et al. 1995). Chaperonin oligomers that were reconstituted in vitro from purified chloroplast cpn60 α and β subunits ($\alpha_7\beta_7$ ch-cpn60) behaved similarly to GroEL and to ch-

cpn60 that was purified in oligomeric form, in terms of refolding heterologous Rubisco (Dickson et al. 2000). Alpha subunits on their own were unable to assemble into functional chaperonin oligomers either in vivo or in vitro (Cloney et al. 1992; Dickson et al. 2000). Interestingly, oligomers reconstituted only from β subunits (β_{14} ch-cpn60) were able to refold Rubisco only with the assistance of the mitochondrial cpn10 (mt-cpn10) in vitro (Dickson et al. 2000). This preference was also shown to be characteristic of the mammalian mitochondrial cpn60 (mt-cpn60; Viitanen et al. 1992, 1998). Presently, the basis for this specificity is not fully understood. It seems likely that the lack of chaperonin activity for β_{14} ch-cpn60 and mt-cpn60 in the presence of plant and bacterial cpn10's reflects an absence of binding between chaperonin and co-chaperonin. However, no direct study has been carried out in the past to confirm this hypothesis. Thus, our primary motivation for carrying out this study was to investigate the binding of various cpn10 homologs to various types of cpn60 oligomer. To this end, a direct binding assay was developed utilizing radioactive co-chaperonins and cross-linking in order to quantitate the binding of cpn10 homologs to cpn60 oligomers. Surprisingly, our results show that both $\alpha_7\beta_7$ ch-cpn60 and β_{14} ch-cpn60 were equally capable of binding cpn10 from bacteria, chloroplast, or mitochondria. Moreover, mt-cpn60 was also able to bind the ch-cpn20. Thus, in our systems, the formation of co-chaperonin/chaperonin complexes does not necessarily lead to folding. In some cases, the association can lead to discharge of bound substrate, into solution, as an intermediate that is not able to reach its active form without assistance.

Materials and methods

Proteins

Constructs of $\alpha_7\beta_7$ ch-cpn60 and β_{14} ch-cpn60 were engineered and the proteins purified and reconstituted as previously detailed (Dickson et al. 2000). GroEL and GroES were purified as in Bonshtien et al. (2007). Mt-cpn60 was purified as previously described (Levy-Rimler et al. 2001). Hexahistidine-tagged mt-cpn10 was purified as reported for histidine-tagged ch-cpn20 (Bonshtien et al. 2007).

Ch-cpn20 from spinach (Viitanen et al. 1995, 1998) was used with the ATPase assays. This ch-cpn20 was previously shown to function with chloroplast cpn60 (Viitanen et al. 1995) and GroEL (Viitanen et al. 1998) in refolding denatured Rubisco and mMDH. However, the protein did not have a stable oligomeric structure in solution and the purification protocol was not straightforward (Sharkia et al. 2003). Therefore, we prepared a new construct, of *A. thaliana* ch-cpn20, which contained a 6-histidine tag at its N terminus and was easily purified. The new ch-cpn20 purified from this

construct was used for binding and refolding assays. Similar to the spinach ch-cpn20, this new protein was fully able to assist both GroEL and chloroplast cpn60 in refolding denatured mMDH in vitro (Bonshtien et al. 2007).

Purification of radiolabeled hexahistidine-tagged ch-cpn20 and mt-cpn10

Overnight cultures grown in dilute Luria broth (LB; 0.25% Bacto Tryptone, 0.125% Bacto yeast extract, and 0.5% NaCl) containing ampicillin (100 µg/ml) were diluted 1:20 into the same medium and grown at 37°C to an OD₆₀₀ of 0.7. Production of radiolabeled his-tagged co-chaperonin was induced by adding 1 mM isopropyl β-D-1 thiogalactopyranoside and 2.5 mCi of Redivue L-[³⁵S] methionine (AG-1094, Amersham Biosciences). Following growth overnight at 37°C, cells were harvested by centrifugation for 10 min at 6,000 rpm in a Sorvall 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 (buffer A) plus protease inhibitor cocktail (1/1,000 dilution of a solution containing 1 mg/ml each of pepstatin, chymostatin, antipain, leupeptin, and aprotinin), 0.5 mM phenylmethanesulphonylfluoride, and 1,500 U/ml DNase I (Sigma). The resuspended cells were sonicated for a total of 3.5 min with 15-s breaks every 15 s. Debris was removed by centrifugation for 1 h at 16,000 rpm in a Sorvall centrifuge in a SS34 rotor. Supernatant was diluted two times in buffer A and bound to Ni-agarose resin, washed three times with buffer A, and eluted with buffer A containing 300 mM imidazole. Relevant fractions were concentrated and desalted (PD10 columns, Pharmacia) with buffer containing 20 mM Na-HEPES, pH 7.4, 200 mM NaCl, and 5% glycerol.

Purification of radiolabeled GroES

Pre-cultures of bacteria overexpressing GroES were grown in diluted LB containing ampicillin (100 µg/ml) at 37°C. Overnight cultures were diluted 1:10 into the same medium and grown at 37°C until an OD₆₀₀ of 0.7. Production of radiolabeled GroES was initiated by addition of 1 mM isopropyl β-D-1 thiogalactopyranoside and 2.5 mCi of Redivue L-[³⁵S] methionine (catalog number AG-1094, Amersham Biosciences). Overexpression took place for 3 h at the same temperature. Cells were harvested by centrifugation for 10 min at 6,000 rpm in a SLC-4000 rotor. The rest of the purification procedure was carried out as for GroES in Bonshtien et al. (2007).

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 mitochondrial malate dehydrogenase

Unfolding of pig heart mMDH (50 µM) was carried out at room temperature in 5 mM HCl. After 1-h incubation, the activity of mMDH was measured to confirm the loss of activity. Following unfolding, mMDH was rapidly diluted (to 0.5 µM) into a solution, pre-warmed to 30°C, containing 15 µM mt-cpn60 or β₁₄ ch-cpn60, 5 mM dithiothreitol (DTT), 10 mM MgCl₂, 50 mM KCl, and 50 mM Na-HEPES, pH 7.4. The refolding of mMDH was initiated by diluting the cpn60-mMDH complex into a half volume of room temperature buffer containing 45% glycerol (where indicated), 60 µM of various co-chaperonins, 6 mM ATP, 50 mM Na-HEPES, pH 7.4, 5 mM DTT, 10 mM MgCl₂, and 50 mM KCl. In some experiments, after 30 min, 20 µM of either mt-cpn10 or 20 mM Na-HEPES, pH 7.4, was added to samples. At various time points, aliquots were removed and assayed for mMDH activity by adding a reaction mixture containing 150 mM potassium phosphate, pH 7.6, 10 mM DTT, 0.28 nM nicotinamide adenine dinucleotide (reduced), and 0.5 mM oxaloacetate. mMDH activity was determined by monitoring the oxidation of nicotinamide adenine dinucleotide (reduced) as a function of time at 340 nm (Badcoe et al. 1991). Refolding yields are expressed as percentage activity of an equal amount of native mMDH.

ATP hydrolysis

This was carried out as previously described (Diamant et al. 2000).

Direct co-chaperonin binding assays

Radioactive co-chaperonin (10 µM) was incubated for 5 min at 30°C with increasing concentrations of either α₇β₇ ch-cpn60 or β₁₄ ch-cpn60 in the presence of 50 mM Na-HEPES, pH 7.4, 100 mM KCl, 20 mM MgCl₂, and 2 mM ATP. Cross-linking was initiated by the addition of 0.1% glutaraldehyde, and the samples were incubated for additional 15 min at 30°C. The reaction was stopped by adding SDS sample buffer containing 1 M urea and subsequent boiling for 5 min. In another variation of this experiment, either α₇β₇ ch-cpn60 or β₁₄ ch-cpn60 was incubated in the presence of increasing concentrations of radioactive co-chaperonin. Cross-linked products were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using an acrylamide gradient of 2.4–12%, which was subsequently dried and exposed to film to visualize the radioactive co-chaperonin. Densitometric analysis was carried out using Image Master ID Prime (GE Healthcare).

Results

ATP hydrolysis by chloroplast chaperonin oligomers—the effect of protein concentration and co-chaperonins

The ATPase activity of GroEL has been extensively studied and well characterized (for a review, see Horovitz et al. 2001). In contrast, little has been done to characterize the ATPase activity of ch-cpn60. Despite the high homology between ch-cpn60 and GroEL, ch-cpn60 is known to be highly unstable at dilute concentrations in the presence of nucleotide (Musgrove et al. 1987; Viitanen et al. 1995, 1998). Therefore, it was interesting to study ATP hydrolysis by the chloroplast homologs as well as the effect of different cpn10s on this activity. As shown in Fig. 1a, at low chaperonin concentrations, ch-cpn60s have a very low rate of ATP hydrolysis ($\sim 0.2 \text{ min}^{-1}$). Upon increasing the concentration of ch-cpn60, the rate of ATP hydrolysis increases up to a maximum of about 0.62 min^{-1} at $60 \mu\text{M}$ protein for $\alpha_7\beta_7$ ch-cpn60 (top panel) and about 0.85 min^{-1} for β_{14} ch-cpn60 (bottom panel). These results suggest that ch-cpn60 in solution exists in equilibrium between monomeric and oligomeric forms, the sub-tetradecameric forms having a lower ATPase activity than the full oligomeric species. Increasing the cpn60 concentration shifts the equilibrium toward the tetradecameric form, causing an apparent increase in the rate of ATP hydrolysis, which reaches a maximum at $60 \mu\text{M}$ chaperonin. Presumably, this concentration reflects that at which most chaperonin molecules are present in solution as tetradecamers. In support of these results, cross-linking of cpn60 oligomers in the presence of ATP showed significant concentration-dependent dissociation of the oligomers (not shown).

The maximal ATP hydrolysis rate obtained is slightly higher for β_{14} ch-cpn60 than $\alpha_7\beta_7$ ch-cpn60 (Fig. 1a). Nevertheless, the dependence of the ATP hydrolysis rate on protein concentration was similar for both β_{14} ch-cpn60 and $\alpha_7\beta_7$ ch-cpn60 oligomers (EC_{50} of $40 \mu\text{M}$ and plateau at $60 \mu\text{M}$ protein), suggesting that both types of oligomers exhibit a similar oligomer stability in the presence of ATP.

Next, we examined the effect of ch-cpn20 on the ATPase activity of both β_{14} ch-cpn60 and $\alpha_7\beta_7$ ch-cpn60 oligomers. In the case of $\alpha_7\beta_7$ ch-cpn60, an interesting phenomenon is observed; ch-cpn20 seems to stimulate ATP hydrolysis at low ch-cpn60 concentrations, while at high concentrations, the rate of ATP hydrolysis is inhibited by slightly over 50%, as described for GroEL (Horovitz 1998; Todd et al. 1993; Fig. 1a, top panel). This can be explained by the fact that co-chaperonin exerts two different effects on the $\alpha_7\beta_7$ ch-cpn60 oligomer. At low protein concentrations, the presence of ch-cpn20 facilitates association of the ATP-hydrolyzing oligomer. At high concentrations of

Fig. 1 **a** ATPase activity of chloroplast cpn60 oligomers. ATP hydrolysis by $\alpha_7\beta_7$ ch-cpn60 (top panel) and β_{14} ch-cpn60 (bottom panel) oligomers was determined as a function of cpn60 concentration in buffer containing 50 mM Na-HEPES, 10 mM MgCl_2 , and 50 mM KCl, 5 mM phosphoenolpyruvate, 20 $\mu\text{g/ml}$ pyruvate kinase, and 2 mM ATP in the presence or absence of a saturating concentration of ch-cpn20. **b** The effect of different co-chaperonin homologs (120 μM) on ATP hydrolysis by ch-cpn60 (60 μM) was determined in the same buffer

$\alpha_7\beta_7$ ch-cpn60, most of this protein is tetradecameric and ch-cpn20 inhibits its ATP hydrolysis by 50%, as observed for other chaperonin homologs. In the case of β_{14} ch-cpn60, no such stabilization or inhibition is induced by ch-cpn20, suggesting that it does not bind this co-chaperonin (Fig. 1a, bottom panel).

The effect of other cpn10 homologs on ATP hydrolysis by $\alpha_7\beta_7$ ch-cpn60 and β_{14} ch-cpn60 was also determined. Only the mitochondrial cpn10 was able to inhibit the ATPase activity of β_{14} ch-cpn60 (Fig. 1b), while ch-cpn20,

mt-cpn10, and GroES all inhibited ATP hydrolysis by $\alpha_7\beta_7$ ch-cpn60 by approximately 50%. These results are consistent with Dickson et al. (2000) who showed that reconstituted $\alpha_7\beta_7$ ch-cpn60 was able to refold denatured Rubisco with the help of cpn10 from any source, while all β_{14} ch-cpn60 oligomers were only functional with cpn10 from mitochondria. This phenomenon was observed when mMDH was used as a substrate as well, suggesting that this specificity is not unique to Rubisco (not shown).

Binding of cpn10 homologs to reconstituted cpn60 oligomers

Protein refolding experiments from previous studies and the ATP hydrolysis assays from this study suggest that $\alpha_7\beta_7$ ch-cpn60 oligomers interact with all co-chaperonins examined here, while β_{14} ch-cpn60 oligomers interact only with mitochondrial cpn10 (Dickson et al. 2000). In order to establish a structural basis for these observations, we performed direct binding assays between chaperonins and various co-chaperonins. Increasing concentrations of reconstituted cpn60 oligomers were incubated with radiolabeled cpn10 homologs in the presence or absence of ATP to allow complex formation. Next, complexes were stabilized by cross-linking with glutaraldehyde. Finally, complexes were separated on denaturing polyacrylamide gradient gels in order to separate free radiolabeled cpn10 from that bound to cpn60 oligomers. The relative amount of cpn10 bound to each type of cpn60 oligomer was determined by densitometry of autoradiograms (Slutsky-Leiderman et al. 2007).

As mentioned above, GroEL and chloroplast $\alpha_7\beta_7$ ch-cpn60 are able to functionally bind GroES, mt-cpn10, and ch-cpn20 (Dickson et al. 2000; Viitanen et al. 1995). Consistent with this, we observed ATP-dependent binding of all the radioactive co-chaperonins to reconstituted $\alpha_7\beta_7$ ch-cpn60, which was concentration-dependent (Fig. 2). Interestingly, reconstituted β_{14} ch-cpn60 was also able to bind all types of co-chaperonins in an ATP-dependent manner (Fig. 2). This result is surprising, since GroES and ch-cpn20 can assist in refolding denatured Rubisco and mMDH, under stringent conditions, only with $\alpha_7\beta_7$ ch-cpn60 and not with β_{14} ch-cpn60 (Dickson et al. 2000).

Fig. 2 Binding of radiolabeled co-chaperonins to ch-cpn60 oligomers. Radiolabeled co-chaperonin (10 μ M) was incubated with increasing concentrations of cpn60 oligomer in the presence or absence of 2 mM ATP. The complex was cross-linked with glutaraldehyde (0.1%) and separated from free co-chaperonin on 2.4–12% gradient SDS-PAGE. Gels were dried and exposed to film for visualization of bound and free co-chaperonin. **a** [35 S] mt-cpn10. **b** [35 S] ch-cpn20. **c** [35 S] GroES. The bands labeled as “bound” represent heptameric (faster mobility) and tetradecameric (slower mobility) oligomeric species and appear due to faster cross-linking within the heptameric rings than cross-linking between the rings (Azem et al. 1994, 1998)

Densitometric analysis of the results in Fig. 2 highlights two observations, both of which are worthy of closer inspection. Firstly, binding of the different cpn10 homologs exhibited similar concentration dependence for association with both $\alpha_7\beta_7$ ch-cpn60 and β_{14} ch-cpn60, reflecting a

similar extent of binding (Fig. 3, left panel). The second observation is that β_{14} ch-cpn60 consistently bound ~50% less GroES than $\alpha_7\beta_7$ ch-cpn60 (Fig. 3, GroES, right panel).

Due to the functional similarity between β_{14} ch-cpn60 and mt-cpn60 in terms of their specificity for the mitochondrial cpn10, it was very interesting to examine whether mt-cpn60 is also able to bind heterologous cpn10s. When the co-chaperonin binding assay was carried out with mt-cpn60, a unique binding pattern was observed. Although this oligomer functions only with mt-cpn10, significant ATP-dependent binding was also observed with ch-cpn20 (Fig. 4a). However, GroES was unable to bind mt-cpn60 under similar conditions, serving as an important control for our system (Fig. 4b). Table 1 summarizes the variety of interactions that can take place between cpn10 homologs and cpn60 oligomers. Clearly, binding is a prerequisite for further function. However, chaperonins can sometimes

form a complex, which does not lead to productive folding (e.g., GroES and β_{14} ch-cpn60; ch-cpn20 and mt-cpn60).

Fate of the bound substrate protein with different co-chaperonin homologs

We have seen that β_{14} ch-cpn60 oligomer is able to bind ch-cpn20 and GroES and that mt-cpn60 can bind to ch-cpn20. Yet, in protein-folding experiments, these interactions do not result in active substrate protein. One possibility is that the folding protein is bound to cpn60 irreversibly. Alternatively, it is possible that the substrate protein reversibly rebound cpn60 or was released into solution and aggregated. We carried out a number of protein-refolding experiments to explore the fate of the substrate protein bound to mt-cpn60 and β_{14} ch-cpn60. Since, native mMDH tends to aggregate under conditions commonly used for protein-refolding assays (Schmidt et al. 1994), we carried out

Fig. 3 Quantification of [35 S]cpn10 binding to ch-cpn60. Bands in the auto radiographs from Fig. 2 were quantified using Image Master ID Prime to give a graphic depiction of the amount of cpn10 bound to ch-cpn60. Empty symbols, $\alpha_7\beta_7$ ch-cpn60; filled symbols, β_{14} ch-cpn60.

Left panel, 100% for each experiment is taken as the maximum binding measured in that experiment. *Right panel*: 100% taken for all experiments is the maximum binding of cpn10 to $\alpha_7\beta_7$ ch-cpn60

Fig. 4 Binding of radiolabeled co-chaperonins to mt-cpn60 oligomers. *Left panel*, experiments were carried out as described in Fig. 2 with either [³⁵S] ch-cpn20 (*top*) or [³⁵S] GroES (*bottom*). *Right panel*, the experiments were carried out with increasing concentrations of co-chaperonin and 10 μM cpn60. The bands labeled as “bound”

represent heptameric (faster mobility) and tetradecameric (slower mobility) oligomeric species and appear due to faster cross-linking within the heptameric rings than cross-linking between the rings (Azem et al. 1994, 1998)

our experiments at permissive temperature, in the presence and absence of glycerol, in order to allow for the recovery of the activity of substrate intermediates released from cpn60. In one set of experiments, binary complex of cpn60 and unfolded substrate was incubated in the presence of different cpn10 homologs with or without 15% glycerol. At various times following the addition of ATP and co-chaperonin, samples were taken for activity determination. When mt-cpn10 was present with ATP upon initiation of refolding, significant refolding of mMDH was observed in the presence or absence of glycerol for both types of cpn60 oligomer (Fig. 5a, b). When refolding was performed in the presence of ch-cpn20, a small recovery of mMDH activity was observed for β₁₄ ch-cpn60 (~35%), while none was observed for mt-cpn60 (<5%). Remarkably, when the latter experiments were carried out in the presence of glycerol, a significant increase in the yield of refolded mMDH was observed, in some cases reaching levels similar to those obtained with mt-cpn10. Using GroES as a co-chaperonin, no mMDH activity was recovered in the absence of glycerol

for both oligomers. However, in the presence of glycerol, activity was regained in the β₁₄ ch-cpn60 system, but not the mt-cpn60 system. These results, together with the binding data, strongly support the idea that the binding of ch-cpn20 to β₁₄ ch-cpn60 and mt-cpn60, as well as the binding of GroES to β₁₄ ch-cpn60, indeed facilitates release of the bound substrate protein from chaperonin. However, the protein is released in a form that is unable to reach a functional conformation. When glycerol is present in solution, it serves as a chemical chaperone and helps the released protein to fold properly.

If the substrate protein is released in a partially unfolded conformation, it should rebind to the cpn60 oligomer and be available for refolding upon addition of mt-cpn10. In order to test this hypothesis, binary complex of unfolded mMDH was formed with either β₁₄ ch-cpn60 or mt-cpn60, and the complex was subsequently incubated with ATP and one of the three co-chaperonins (mt-cpn10, ch-cpn20, or GroES). After 30 min, mt-cpn10 was added to all of the samples and mMDH activity was measured after another

Table 1 Differential effects of co-chaperonins on cpn60 oligomers

	Refolding ^a	ATPase inhibition	Binding
mt-cpn10			
GroEL	+	+	+
$\alpha_7\beta_7$ ch-cpn60	+	+	+
β_{14} ch-cpn60	+	+	+
mt-cpn60	+	+	+
ch-cpn20			
GroEL	+	+	+
$\alpha_7\beta_7$ ch-cpn60	+	+	+
β_{14} ch-cpn60	–	–	+
mt-cpn60	–	ND	+
GroES			
GroEL	+	+	+
$\alpha_7\beta_7$ ch-cpn60	+	+	+
β_{14} ch-cpn60	–	–	+
mt-cpn60	–	–	–

^aUnder stringent conditions; Viitanen et al. (1992, 1995, 1998), Dickson et al. (2000)

120 min. As a control, an identical set of samples was prepared, to which no mt-cpn10 was added. It was found that the addition of mt-cpn10 to the samples induced full recovery of mMDH activity in all cases for both β_{14} ch-cpn60 and mt-cpn60 (not shown). We conclude that the association of ch-cpn20 with mt-cpn60 and β_{14} ch-cpn60, or of GroES with β_{14} ch-cpn60 oligomers, induces dissociation of bound substrate into solution in a form that is not folding-competent. The unfolded substrate then rebinds cpn60 and is available for refolding when mt-cpn10 is added.

Discussion

Although their *in vitro* structure and function seemed at first to be very much like the well-studied bacterial chaperonins, it has become apparent that there are significant differences in both the structure of organellar chaperonins and the way they function. Such differences include the oligomeric instability of organellar homologs, the multiplicity of chloroplast chaperonin homologs, and the high specificity that the mitochondrial cpn60 exhibits for its endogenous co-chaperonin. All these homologs are able to refold denatured substrate protein *in vitro*, yet exhibit distinct structural and functional characteristics, therefore providing a unique tool with which to explore various aspects of the chaperonin refolding cycle.

Studies of ch-cpn60 have been hampered in the past by excessive dissociation of the oligomer in the presence of

ATP at concentrations commonly used for biochemical studies (Hemmingsen and Ellis 1986; Musgrove et al. 1987; Viitanen et al. 1995, 1998). Indeed, initial *in vitro* refolding studies of purified chloroplast cpn60 showed that under conditions of the refolding assay, 35% of the chaperonin oligomer dissociated to monomer (Viitanen et al. 1995). However, significant reconstitution of chloroplast ch-cpn60 oligomer was achieved from monomers *in vitro*, in the presence of ATP, at protein concentrations of 60 μ M (Lissin 1995), and maximal reconstitution of β_{14} ch-cpn60 oligomers from monomeric starting material *in vitro* was achieved at about 200 μ M β monomer in the presence of Mg^{2+} -ATP (Dickson et al. 2000). It is generally agreed that organellar chaperonin oligomers are unstable at low concentrations in the presence of ATP. It was therefore interesting to analyze ATP hydrolysis by both types of reconstituted ch-cpn60 oligomer as a function of chaperonin concentration. In the absence of cpn10 homologs, there was a concentration-dependent increase in turnover rate, which reached a plateau at approximately 60 μ M chaperonin, indicating that at this

Fig. 5 Refolding of mMDH in the presence or absence of 15% glycerol. A binary complex was formed between urea-denatured mMDH and either mt-cpn60 (*top panel*) or β_{14} ch-cpn60 (*bottom panel*) at 30°C as described in “Materials and methods”. mMDH activity was then determined at room temperature at various time points following the addition of co-chaperonin and ATP either in the presence or absence of glycerol. mt-cpn10 (*filled circle*); mt-cpn10 with glycerol (*empty circle*); ch-cpn20 (*filled square*); ch-cpn20 plus glycerol (*empty square*); GroES (*filled triangle*); GroES plus glycerol (*empty triangle*); no co-chaperonin (*ex*); no co-chaperonin with glycerol (*plus symbol*)

concentration, both types of cpn60 are fully assembled into active oligomers. This conclusion is supported by cross-linking experiments of various concentrations of cpn60 that showed a concentration-dependent dissociation in the presence of ATP. These results also suggest that the monomeric form of ch-cpn60 is much less active than the oligomer, if at all. Thus, β_{14} ch-cpn60 and $\alpha_7\beta_7$ ch-cpn60 tetradecamers have the same stability when diluted into solution in the presence of ATP. For both oligomer types, a turnover number of less than 1 min^{-1} was measured ($0.6\text{--}0.8 \text{ min}^{-1}$). A similar rate was reported for mammalian mitochondrial cpn60 (Levy-Rimler et al. 2001), for ch-cpn60 from narcissus (Bonk et al. 1996), and for GroEL that was super-purified from all contaminating peptides (Todd and Lorimer 1998; G. Lorimer, personal communication). These values may reflect the fact that the purification method used in this study produces highly pure chaperonin preparations that contain no contaminating peptides.

In the presence of ch-cpn20, a different picture is obtained. At low $\alpha_7\beta_7$ ch-cpn60 concentrations, the presence of ch-cpn20 causes an apparent increase in turnover number relative to the system lacking co-chaperonin. This can be explained by the stabilizing effect exerted by co-chaperonins on cpn60 oligomer, on the one hand, and by the fact that protein monomers are much less active (Dickson et al. 2000; Horovitz et al. 1993; Lissin 1995). At high protein concentrations, under which conditions the protein is primarily tetradameric, its ATP hydrolysis is inhibited by $\sim 50\%$ in the presence of cpn10 homologs, similar to GroEL. Such inhibition was not observed for β_{14} ch-cpn60 with ch-cpn20 or GroES, co-chaperonins with which it is not functional (Dickson et al. 2000). It should be noted that the ATPase of mt-cpn60 was inhibited by mt-cpn10 at all concentrations of cpn60 tested. However, at low protein concentrations, the inhibition was less pronounced; only at concentrations above $10 \text{ }\mu\text{M}$ was the full 50% inhibition observed, suggesting that the oligomeric form of mt-cpn60 is also sensitive to dilution in the presence of ATP (Levy-Rimler et al. 2001).

Similar to the mammalian mitochondrial cpn60, reconstituted β_{14} ch-cpn60 oligomers can refold denatured Rubisco and mMDH under stringent conditions only with the help of mt-cpn10, indicating that this form of oligomer is not the predominant form in vivo. Indeed, one report showed that chloroplast chaperonin oligomers could be precipitated with antibodies to either α or β subunits, suggesting that the natural form contains both subunit types (Nishio et al. 1999). However, the ability to reconstitute an oligomer of chloroplast origin, which functions with similar specificity to the mitochondrial homolog, provides us with a unique system that can be used to probe structural characteristics of chaperonins.

Despite the lack of functionality, we show clear binding of all the cpn10 homologs to β_{14} ch-cpn60. Although we found this result surprising at first, Dickson et al. (2000) presented data showing a mild stimulation of β -subunit oligomerization by both GroES and ch-cpn20, indicating that some weak binding was taking place. Even more interesting are the results of co-chaperonin binding to mt-cpn60: there was clear binding of mt-cpn10 and ch-cpn20, but not of GroES. The fact that GroES was unable to bind serves as an important control for our experimental system and shows that the binding observed in this study is indeed specific.

The mMDH refolding experiments that we carried out in the absence and presence of glycerol show that we can divide the outcome of chaperonin/co-chaperonin interactions into two types. The first type leads to discharge of bound substrate protein into solution in a folding-competent form. The second type allows for release of bound substrate into solution as folding-incompetent forms that cannot fold without assistance. A relatively simple explanation can be put forth to explain these observations. The literature speaks about an initial docking step of cpn10 on cpn60, one in which the cpn10 oligomer binds cpn60 but does not induce the large conformational changes that are required for formation of the folding chamber or “anfinen cage” (Chapman et al. 2008; Ranson et al. 2001). Additionally, cpn10 and substrate protein partially compete for a binding site on cpn60 (Fenton et al. 1994). Thus, when we see binding of cpn10 that does not correlate with protein refolding, the binding may only reflect the initial docking step. Instead of the substrate protein entering into the folding chamber (which does not form), it is displaced by cpn10 and released into the surrounding medium, only to be rebound due to the fact that it is still not folded properly. These forms rebound cpn60 and are available for functional refolding when the proper conditions are provided.

The primary mediator of cpn10 binding to cpn60 was shown to be a “mobile loop,” a highly flexible sequence of about 18 amino acids which becomes more rigid and adopts a β -hairpin structure upon binding to GroEL (Landry et al. 1993, 1996). A highly conserved tripeptide of “IVL” in the GroES mobile loop was proposed to mediate binding (Landry et al. 1993, 1996). The mobile loop of mt-cpn10 is similar to that of GroES with a number of critical differences. In mt-cpn10, the middle amino acid of the

GroES <i>E. coli</i>	EVETKSAGGIVLTGSAA
N-ch-cpn20 <i>Arath</i>	EAEKTLGGILLPSTAQ
C-ch-cpn20 <i>Arath</i>	EAEKTAGGLLLTETTK
mt-cpn10 <i>Mouse</i>	AAETVTKGGIMLPKESQ

Fig. 6 Comparison of the mobile loop sequence of various cpn10 homologs

tripeptide is methionine, which was suggested to be more hydrophobic than the corresponding valine of GroES. In addition, a proline residue that follows the tripeptide in the mt-cpn10 homolog (corresponding to threonine 28 in GroES) was suggested to reduce conformational dynamics of the loop and to favor the chaperonin-bound conformation, while a threonine 4 amino acids before the tripeptide in mt-cpn10 (corresponding to serine 21 in GroES) was thought to increase the tendency of the area to form a β -sheet compared with the bacterial serine at the same position (Richardson et al. 2001).

The mobile loop of the N-terminal domain of ch-cpn20 is similar to mammalian cpn10 in conservation of both the threonine before the tripeptide and the proline following it, perhaps explaining its ability to bind both β_{14} ch-cpn60 and mt-cpn60 (Fig. 6). Indeed, it was shown that changing S21>T and T28>P of GroES is enough to impart partial activity to GroES working with mt-cpn60, indicating that binding occurs in this mutant (Richardson et al. 2001). It is noteworthy that when the middle amino acid of the tripeptide in GroES is changed from valine to methionine and the following threonine is changed to proline, full activity is conferred on GroES with mt-cpn60 (Bonshtien, data not shown). Interestingly, this latter mutant did not bestow on GroES the ability to function with β_{14} ch-cpn60 (Bonshtien, data not shown), further emphasizing the differences between the various types of chaperonin homologs and their co-chaperonin specificities. Clearly, further studies are required in order to determine more specifically the molecular interactions that define and govern cpn10 binding to cpn60.

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