Chaperone Activity of a Chimeric GroEL Protein That Can Exist in a Single or Double Ring Form*

Annette Erbse‡, Ofer Yifrach§, Susan Jones‡, and Peter A. Lund‡

From the ‡School of Biological Sciences, University of Birmingham, Birmingham B15 2TT, United Kingdom, the §Department of Structural Biology, Weizmann Institute of Science, Rehovot 76100, Israel, and the ¶School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, United Kingdom

The molecular chaperone GroEL is a protein complex consisting of two rings each of seven identical subunits. It is thought to act by providing a cavity in which a protein substrate can fold into a form that has no propensity to aggregate. Substrate proteins are sequestered in the cavity while they fold, and prevented from diffusion out of the cavity by the action of the GroES complex, that caps the open end of the cavity. A key step in the mechanism of action of GroEL is the transmission of a conformational change between the two rings, induced by the binding of nucleotides to the GroEL ring opposite to the one containing the polypeptide substrate. This conformational change then leads to the discharge of GroES from GroEL, enabling polypeptide release. Single ring forms of GroEL are thus predicted to be unable to chaperone the folding of GroES-dependent substrates efficiently, since they are unable to discharge the bound GroES and unable to release folded protein. We describe here a detailed functional analysis of a chimeric GroEL protein, which we show to exist in solution in equilibrium between single and double ring forms. We demonstrate that whereas the double ring form of the GroEL chimera functions effectively in refolding of a GroES-dependent substrate, the single ring form does not. The single ring form of the chimer, however, is able to chaperone the folding of a substrate that does not require GroES for its efficient folding. We further demonstrate that the double ring structure of GroEL is likely to be required for its activity in vivo.

The chaperonins or HSP60 proteins are essential for cell growth (1). In vivo, they assist in the folding of newly synthesized or stress denatured proteins (for recent reviews, see Refs. 2–4). The chaperonin GroEL from Escherichia coli is the best characterized member of the chaperonin family. It facilitates protein folding in vivo together with its protein co-factor GroES. GroEL has 14 identical 57-kDa subunits, organized in two heptameric rings stacked back to back with a central cavity in each ring. The arrangement of the subunits and the size of the cavity change dramatically in the presence of nucleotide and GroES (5–9). Protein folding is thought to take place within this cavity. Detailed in vitro studies of the ATPase activity of GroEL, and GroEL-mediated protein refolding, suggest that the double ring structure and the transmission of allosteric information between the rings are key features of the mechanism by which the chaperone acts. Unfolded or partially folded substrate protein is thought to bind to the apical region of one of the two rings. The subsequent binding of ATP and GroES to the same ring induces a structural reorientation of the subunits, creating a cis-complex where the bound protein has been discharged into the central cavity, which is sealed by GroES. The protein folds in this cavity, where unfavorable hydrophobic interactions with other unfolded proteins are prevented. The protein remains trapped by the GroES co-factor until the binding of ATP to the opposite (trans) ring releases GroES. This binding cannot take place until the slow hydrolysis of ATP on the cis ring has occurred, which also primes the GroES for release (10). In vitro, GroEL can assist the refolding of some substrate proteins without the aid of GroES. The mechanism of GroES independent refolding is not clearly understood.

ATP binding and hydrolysis is an intrinsic part of the mechanism by which GroEL functions. GroEL shows two levels of cooperativity for the binding and hydrolysis of ATP: positive cooperativity between the subunits of each ring and negative cooperativity between the two rings (11–14). According to the model of nested cooperativity (12–14), the subunits in each ring are in equilibrium between a T-state (with high affinity for unfolded polypeptides, and low affinity for ATP) and an R-state (with low affinity for protein and high affinity for ATP). In the absence of nucleotide the subunits of both rings are mostly in the T-state, forming a symmetrical TT-state. Binding of ATP to one ring induces a concerted transition of all the seven subunits in that ring to an R-state, while those in the other ring remain in the T-state, resulting in an asymmetrical TR form. With increasing ATP concentration the negative cooperativity between the rings is overcome and the binding of ATP to the second ring induces a second transition so that a symmetrical R'R'-state is reached. The designation R' indicates that the catalytical properties of the R-states in the TR and R'R' form are not identical.

The role of the double ring structure has been examined by several groups, using a single ring GroEL mutant (10, 15–17) and “mini-chaperones” (consisting of the apical protein-binding domains alone from GroEL) (18–21). According to the model described above, a single ring form of GroEL should not show GroES-dependent chaperone activity, as the bound GroES would never be released and the substrate protein would stay sequestered beneath it. Studies on a single ring mutant of GroEL have indeed shown this to be the case (10). It was shown that although proteins continue to fold in the cavity of the single ring after ATP and GroES binding, they cannot be released except under special experimental conditions like a cold shock or high salt concentrations (15–17). It was also shown that the single ring GroEL cannot function in vivo (22). This provides strong evidence for the above model, but it could be
argued that because a number of mutations have to be introduced to obtain single rings (15), these mutants may be defective in chaperone function for other unknown reasons. It has also been shown that mini-chaperones cannot mediate the refolding of GroES-dependent substrates, again reinforcing the requirement for the complete double ring structure for GroEL/GroES-mediated folding of proteins. Only in the case of mammalian mitochondrial Hsp60 is there convincing evidence that the protein can act as a chaperone when in the single ring form. However, it can only interact with its own Hsp10 and not with GroES, indicating that there may be quite a distinct mechanism for chaperone activity in this case (23).

We recently described the construction of a series of chimeric GroEL proteins (24), and reported that a number of these proteins were able to support growth as the sole GroEL protein in *Escherichia coli* but nevertheless appeared to exist as single rings when analyzed by native gel electrophoreses. A single amino acid residue crucial for ring-ring interactions in most GroEL homologues was identified, and mutation of this residue enabled us to obtain pairs of GroEL proteins differing only at this position but with very different mobilities on native polyacrylamide gels. We report here the detailed characterization of one of these pairs of chimeras and show that, in further support of the above model, the single ring form of the chimeric protein cannot chaperone the folding of a GroES-dependent substrate, whereas the double ring form can. The folding of a GroES independent substrate, however, is not affected by the state of oligomerization of the two rings.

**EXPERIMENTAL PROCEDURES**

**Growth of Bacterial Strains and Complementation Analysis—**E. coli strains were grown in L-broth with kanamycin (50 mg/ml), carbenicillin (200 mg/ml), and (unless otherwise indicated) 0.2% (w/v) arabinose. Chimeric groEL genes were created and expressed from the pBAD promoter as described (24). Chromosomal groEL genes were deleted by P1 transduction from a strain that had a precise replacement of the groEL gene with the pntII gene from *S. typhimurium* (25). Strains carrying ΔgroEL::pntII are referred to as AI90 plus the name of the complementing plasmid. Complementation was tested in these strains and in SF103, which was made by P1 transduction of the groEL44 allele from *C. songarae* (26) into *E. coli* strain SF103 (27). SF103 does not grow at 42 °C unless functional GroEL is expressed from a plasmid. To assay complementation, overnight cultures of strains with a groEL44 or a ΔgroEL genotype, containing plasmids expressing wild type or chimeric groEL genes, were diluted, spotted onto plates, and incubated at 37 and 44 °C.

**Proteins—**Bovine lactate dehydrogenase (LDH) and porcine heart mitochondrial malate dehydrogenase (mMDH) were obtained from Sigma. *E. coli* GroES was a generous gift from G. R. Clarke (University of Bristol). GroEL and GroEL chimeric proteins were isolated as described by Yifrach and Horovitz (12) from strains expressing P211 as the sole GroEL protein from the pBAD promoter in a ΔgroEL background (24). 15% methanol was added to samples before fractionation on a Q-Sepharose high performance column (Pharmacia), equilibrated with storage buffer (50 mM Tris, pH 7.5, 60 mM KCl, 20 mM MgCl₂, 0.1 mM EDTA, and 5 mM DTT) and snap frozen in liquid nitrogen. Aliquots were stored at −80 °C. Chaperonin concentration was determined according to Bradford (27) using defatted bovine serum albumin as standard. GroES concentration was measured using the extinction coefficient of 3440 molar⁻¹ cm⁻¹ for protomers (28). Concentrations of GroEL chimeras are given with respect to tetradecamers, the concentrations of mMDH and LDH with respect to monomers.

**Protein Folding Assays—**Refolding of mMDH was performed as in Ranson et al. (29) with minor modifications. 150 μM mMDH was denatured in 3 M guanidine hydrochloride containing 20 mM DTT at 20 °C for 2 h. Refolding was initiated by dilution of the refolding buffer (150 mM Tris, pH 7.5, 50 mM KCl, 20 mM MgCl₂, 5 mM DTT) to a final concentration of 150 mM. If refolding was measured in the presence of cofactors, the final concentrations were 150 mM chaperonin oligomer (unless otherwise indicated), 2 mM ATP, and a 4-fold excess GroES oligomer to GroEL oligomer. Chaperonins, GroES, and ATP were added to the standard buffer directly before initiating refolding. To measure refolding, aliquots were removed from the refolding mixture and snap frozen in liquid nitrogen. *In vivo* experiments were performed at 30°C. Porcine LDH refolding was measured as described (30) with minor modifications. LDH was denatured in 6 M guanidine hydrochloride (pH 3.2) for 15 min at room temperature. If not indicated otherwise, renaturation was initiated by dilution into buffer (0.1 M Na₃H₂PO₄, pH 7, 1 mM EDTA, 1 mM DTT, 10 mM KCl, and 10 mM MgCl₂) to a final concentration of 150 mM LDH. If LDH refolding was measured in the presence of chaperonins and ATP the final concentrations of chaperonin and ATP were 150 mM (if not indicated otherwise) and 2 mM, respectively. Chaperonins and ATP were added to the buffer directly before the refolding was initiated. Aliquots were taken and added to a solution of 0.2 mM NADH and 2.25 mM pyruvate in 0.1 M phosphate buffer, pH 7. NADH oxidation by active LDH was observed by monitoring the decrease in absorption at 340 nm over 120 s in a Shimadzu UV1601 spectrophotometer. All assays were carried out at 30 °C. Porcine LDH refolding was measured as described (31) with minor modifications. LDH was denatured in 6 M guanidine hydrochloride (pH 3.2) for 15 min at room temperature. If not indicated otherwise, renaturation was initiated by dilution into buffer (0.1 M Na₃H₂PO₄, pH 7, 1 mM EDTA, 1 mM DTT, 10 mM KCl, and 10 mM MgCl₂) to a final concentration of 150 mM LDH. If LDH refolding was measured in the presence of chaperonins and ATP the final concentrations of chaperonin and ATP were 150 mM (if not indicated otherwise) and 2 mM, respectively. Chaperonins and ATP were added to the buffer directly before the refolding was initiated. Aliquots were taken and added to a solution of 0.2 mM NADH and 2.25 mM pyruvate in 0.1 M phosphate buffer, pH 7. NADH oxidation by active LDH was observed by monitoring the decrease in absorption at 340 nm over 120 s in a Shimadzu UV1601 spectrophotometer.

**ATPase Assays—**The ATPase activity of GroEL and the chimeric proteins were measured as described by Horovitz et al. (31).

**Data Analysis—**Initial velocity of ATP hydrolysis as a function of the ATP concentration by wild type GroEL and Cpn60-1 was fitted to Equation 1 derived by Yifrach and Horovitz (13) for a three-state nested cooperativity model,

\[
V_0 = \frac{[S]_{\text{max}}}{1 + [S]_{\text{Km}}^n} [1 + L_1 (1 + [S]_{\text{Km}}^n) + L_2 (1 + [S]_{\text{Km}}^n)^2]
\]

where \(L_1\) and \(L_2\) are the apparent allosteric constants for the transitions TT → TR and TR → R'R', \(K_C\) is the dissociation constant of ATP for the R' form, and \(V_C\) and \(V_T\) are the maximal initial velocities of the TR- and R'R'-states. \(S\) is the ATP concentration. Cooperativity in ATP hydrolysis by P211 at high protein concentration (150 nm) and by P211-T101R were analyzed by directly fitting initial ATPase velocity to the simplified nested cooperativity equation below (12) assuming that the TT-state can be ignored,

\[
V_0 = \text{max} (S) [1 + V_{\text{max}} L_2 [S] + [S]_{\text{Km}}^n] (1 + [S]_{\text{Km}}^n)
\]

with the same parameters as before. Cooperativity in ATP hydrolysis by P211 at low enzyme concentration (50 nm) was analyzed by directly fitting initial ATPase velocity to the Hill equation,

\[
V_0 = \text{max} (S)^n (1 + k[S])
\]

where \(V_C\) and \(V_{\text{max}}\) are the initial and maximal initial ATPase velocity, \(S\) is the ATP concentration, \(K\) is the apparent ATP binding constant, and \(n\) is the Hill coefficient.

**RESULTS**

**In Vivo Properties of the P211 Chimera—**We have used homologous recombination in vivo to generate chimeric GroEL proteins between the GroEL protein of *E. coli* and the GroEL homologue Cnp60-1 from the root nodulating bacterium *Rhizobium leguminosarum*. One such chimeric chaperone, referred to as P211, contains the minimal sequence folding from position 1 to 364, and the sequence from Cnp60-1 from position 365 to 547. As we reported earlier (24), purified P211 protein migrates on native gradient PAGE gels with an apparent molecular mass of 380 kDa (by comparison with native molecular mass markers), unlike GroEL or Cnp60-1, which run with an apparent molecular mass of approximately 800 kDa (Fig. 1).

\[2\] The abbreviations used are: LDH, lactate dehydrogenase; mMDH, mitochondrial malate dehydrogenase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.
A Double Ring Is Needed for GroEL-ES-mediated Folding

This is consistent with P211 running as a single ring. We also purified the mutant P211T101R, which is identical with P211 but with an arginine replacing threonine at position 101 (24). This amino acid is crucial for the formation of a double ring structure in Cpn60-1 and re-estabishes a double ring structure in the chimera (24). Consequently purified P211T101R runs on native gels with the same molecular mass as wild type GroEL (Fig. 1).

To investigate the properties of the chimeric proteins in vivo, we deleted the chromosomal groEL gene from strains where P211, P211T101R, or GroEL were expressed on plasmids from the pBAD promoter, which is activated in the presence of arabinose by the AraC protein (32). The strains expressing P211 and P211T101R grew in liquid media in the presence of arabinose at both 37 and 43 °C (Ref. 24 and data not shown). However, at 43 °C strains expressing P211 grew at approximately half the rate of those expressing P211T101R or wild type GroEL. We carried out further studies monitoring growth of the strains on solid media at different concentrations of inducer. The results (Table I) show that at lower arabinose concentrations (< 0.01%) the growth of the groEL deletion strain expressing P211 (AI90/pBAD211) is reduced and at an arabinose concentration of 0.005% growth is completely abolished, in contrast to the same strains expressing GroEL (AI90/pBAD50) or P211T101R (AI90/pBAD211T101R), which still grow under these conditions. These results indicate that cells require a higher concentration of P211 than they do of GroEL or P211T101R for growth, even under non-heat shock conditions. We also examined the ability of the strains to plate the GroEL-dependent bacteriophage λ using strain SF103 (carrying the groELts44 allele) expressing GroEL, P211, or P211T101R from the groE promoter, together with GroES, on derivatives of the plasmid pMa5.8 (33). Bacteriophage λ plated on SF103/pMa211 with an efficiency of 0.16 relative to the wild type control SF103/pMa50, whereas λ plated on SF103/pMa211T101R with approximately the same efficiency as the wild type control. From these in vivo results we conclude that P211 can function in vivo, but is somewhat less effective than wild type E. coli GroEL protein or P211T101R.

ATP Hydrolysis—The results from the in vivo experiments led us to ask whether the P211 chimera is indeed able to act as a single ring, or whether double rings are required for chaperone activity despite its appearance as a single ring on native gels. We used analysis of the kinetics of ATP hydrolysis by P211 and P211T101R to probe for possible inter-ring communication in the proteins under the same conditions as those used for carrying out protein refolding assays.

The initial velocity of ATP hydrolysis by wild type GroEL as a function of the ATP concentration shows a very characteristic curve (Fig. 2a, inset). Two transitions are observed in this curve, which correspond to the two levels of cooperativity, one
was measured at 2.5 mM ATP (data not shown). The data were directly fitted to the Hill equation. The value of the Hill coefficient for the allosteric transition was found to be 2.04 (± 0.134) indicating positive cooperativity between the seven subunits in a single ring. Thus, at 50 nM P211, no indication of any ring-ring interaction was detectable indicating that P211 exists predominately in a single ring form at this concentration.

Dramatically different results were obtained if the initial velocity of ATP hydrolysis was measured using 150 nM P211 over an ATP concentration range from 0 to 2.5 mM. The resulting curve (Fig. 2B) has two transition points, indicating two levels of cooperativity, even though the overall shape of the curve is very different from the curve for GroEL. The reappearance of a double transition indicates that at high protein concentrations the double ring structure of P211 is restored and allosteric communication between the rings can take place. These findings suggest strongly that P211 exits in equilibrium between a single and a double ring structure, with the double ring form predominating at higher protein concentrations.

The data were then fitted to Equation 2. The allosteric transition constant L2 for the TR to the RR*-state for P211 was found to be 2 (± 0.5) × 10⁻⁶. This is significantly higher than for GroEL where L2 is found to be 6 (± 3.2) × 10⁻⁶; this indicates weaker inter-ring communication in the P211 double ring structure compared with GroEL. The data for ATP hydrolysis by P211 (150 nM) at ATP concentrations from 0 to 600 nM were fitted to the Hill equation. The Hill coefficient was 1.21 (± 0.141) in contrast to a Hill coefficient of 2.04 found for the single transition observed at 50 nM P211. Thus the formation of the double ring structure not only restores inter-ring communication but also leads to a decrease of the cooperativity between the seven subunits of each ring. The values of kcat for the TR and RR*-state obtained from this fit and calculated for 7 and 14 sites, respectively, are 0.0298 (± 0.0015) s⁻¹ and 0.0224 (± 0.0012) s⁻¹. These differ from the kcat values for GroEL for which we found to be 0.141 (± 0.0024) s⁻¹ for the TR and 0.016 (± 0.00056) s⁻¹ for the RR*-state, in good agreement with published data (13). kcat for the TR-state of P211 is significantly lower than for GroEL and of the same order of magnitude as kcat for the RR*-state, which accounts for the different overall shape of the curve.

For comparison, the initial rate of ATP hydrolysis by the P211T101R protein was measured using 25 nM protein and ATP concentrations from 0 to 800 nM. This protein is expected to have a stable double ring structure, on the basis of its behavior on native gels. The resulting curve (Fig. 3) shows two transitions, indicating that P211T101R has a double ring structure even at this low protein concentration. The overall shape of the curve is very similar to that seen for P211 when this protein is in the double ring form. By fitting the data to Equation 2, L2 was estimated to be 3.1 (± 1.1) × 10⁻⁶. As for P211, this is significantly higher than for GroEL, indicating that the cooperativity between the rings in P211T101R is weaker than in GroEL. The values of kcat for the TR and RR*-state obtained from this fit and calculated for 7 and 14 sites, respectively, are 0.0347 (± 0.0014) s⁻¹ and 0.0247 (± 0.0010) s⁻¹.

GroES-dependent Refolding—According to the model described earlier, allosteric signaling between the two rings of GroEL is essential for the GroEL-GroES-dependent refolding of denatured proteins (10). Thus P211 should be able to support the refolding of GroES-dependent substrates in the double ring form but not in the single ring form. To test this hypothesis we investigated the refolding of chemically denatured mMDH at different concentrations of P211. The efficient chaperone-assisted refolding of mMDH requires GroEL, GroES, and MgATP (29, 34, 35). Fig. 4A shows the refolding of mMDH at 25 °C with GroEL. Spontaneous refolding is slow and inefficient with the maximal regain of activity being approximately 15%. Refolding is completely abolished in the presence of GroEL, due to the strong binding of unfolded mMDH to GroEL. Addition of MgATP is not enough to overcome this but on further addition of GroES a maximum of 70% mMDH activity can be regained. We found no difference in yield and rate of this reaction in the presence of 20 or 150 nM wild type GroEL. Fig. 4B shows similar experiments with 150 nM P211, a concentration where it exists predominantly in double ring form as shown by the ATPase assays above. P211 binds denatured mMDH and inhibits spontaneous refolding of the protein completely. The addition of ATP alone can overcome the binding of mMDH to P211, allowing refolding to occur to the same level as spontaneous refolding. This indicates that the binding of mMDH to P211 may be weaker than it is to GroEL. Only in the presence of MgATP and GroES is P211 able to support the refolding of mMDH to a significant level. At 150 nM P211 the yield and rate of the refolding in the presence of MgATP and GroES is very similar to that obtained with GroEL. The same results were found for the P211T101R mutant (Fig. 4B) which was again used as a control.

Fig. 5 shows the results for a similar experiment carried out with different concentrations of P211 and P211T101R. For GroEL (data not shown) and P211T101R, varying the chaperone concentration from 150 to 20 nM had no effect on the GroES-dependent refolding of mMDH. However, P211 clearly loses its chaperone activity as its concentration falls although P211 alone is still able to bind denatured mMDH and inhibit its spontaneous refolding (data not shown). In the presence of GroES, ATP, and 150 nM P211, about 60% mMDH activity can be regained with a half-time of 30 min. If the P211 concentration is lowered the percentage of regained MDH activity falls. At 20 nM P211 only 18% activity can be regained with a half-time of 90 min, which is almost identical to the spontaneous refolding of mMDH. Thus the single rings detected by the ATPase assay are not able to function effectively as chaperones with mMDH as a substrate.

GroES-independent Refolding—Not all substrate proteins require GroES as a cofactor for efficient refolding in vitro. We speculated on the basis of the above model that single ring chaperonins would still mediate GroES independent refolding. We therefore carried out refolding assays with LDH, a GroES-independent substrate for GroEL (36, 37). Guanidine hydrochloride-denatured LDH can refold spontaneously to some ex-
tent upon dilution in refolding buffer. Addition of GroEL to the refolding buffer leads to the loss of refolding, due to trapping of the unfolded LDH. In the presence of MgATP and GroEL, a substantially higher level of activity can be regained than with spontaneous refolding. Fig. 6 shows the refolding of LDH in the presence of 150 nM GroEL or P211 with or without ATP and in the presence of 20 nM P211. Addition of equimolar amounts of P211 to denatured LDH monomers in the absence of ATP leads to a total loss of spontaneous refolding, the same result as observed with GroEL. This indicates that under these conditions, P211 and GroEL can bind LDH with similar efficiencies.

In the presence of MgATP and P211, 55% of the LDH activity can be regained showing that P211 can, in the presence of ATP, support LDH refolding with a rate and yield very similar to GroEL. Furthermore, no difference in the refolding activity of P211 is seen with decreasing P211 concentration.

**DISCUSSION**

Several recent studies have looked at the role of the double ring structure and the allosteric communication between the rings in the functioning of GroEL. These were carried out by comparing the chaperoning ability of wild type GroEL with single ring GroEL mutants (10, 15–17) or isolated apical domains (18–21). They concluded that for GroES-dependent substrates, the double ring is required for GroEL to function as a chaperone. Here we have extended this approach by utilizing a GroEL chimeric protein that can exist in both the single ring and double ring states. We show that its chaperone properties change according to which state it is predominantly in. The chimeric protein P211 migrates with a size consistent with it being a single ring protein on native polyacrylamide gels.
gels, and negative stain EM of purified P211 also revealed only single ring particles. However, as both these techniques can perturb equilibria for multimeric proteins, we used ATPase activity of P211 as a probe for the formation of double ring structures under equilibrium conditions. GroEL is known to have two levels of allosteric interactions, a positive cooperativity in ATP binding and hydrolysis within the seven subunits of one ring and a negative cooperativity between the two rings (12–14). This leads to a characteristic biphasic curve (Fig. 2) for initial ATPase rates ($V_o$) as a function of the ATP concentration.

Unlike GroEL, the initial velocity of ATP hydrolysis by P211 as a function of the ATP concentration follows a simple sigmoid curve if determined at low protein concentration (50 nM). The lack of any evidence for a second transition indicates that at this P211 concentration no inter-ring communication takes place although there is a positive cooperativity between the subunits of one ring. This shows that at P211 exists predominantly in a single ring form at lower protein concentrations. At high P211 concentrations, initial velocity as a function of ATP concentration shows a clear double transition, showing that P211 exists as a double ring at high protein concentrations. These results suggest strongly that P211 exists in a concentration dependent equilibrium between single and double ring forms. In contrast, for P211T101R the curve for $V_o$ as function of the ATP concentration exhibits two transitions even at low protein concentrations confirming that the mutant always has a double ring structure and allosteric inter-ring communication can take place.

The shapes of the curves obtained for P211T101R and for P211 in its double ring form are very similar but strikingly different to that obtained from GroEL. The strongest difference are the lack of a peak in the initial velocity at lower ATP concentration and the increase of $V_o$ at higher ATP concentrations. Using the nested cooperativity model by Yifrach and Horovitz (13) to analyze the data, the results for P211 in its double ring form and for P211T101R are best fitted using Equation 2 (12). This equation was derived for a GroEL mutant for which the initial equilibrium between the TP-, TR-, and R'R'-state is shifted in the absence of nucleotides from the TT- to the TR-state. Consistent with this model, the positive cooperativity in ATP hydrolysis observed with GroEL is nearly totally abolished for P211 in its double ring form and for P211T101R. The significant higher transition constant for the TR to R'R' transition of the chimeric proteins compared with GroEL indicates that the negative cooperativity between the rings is much weaker in the chimeric proteins than in wild type GroEL and the formation of the R'R'-state is much more favored.

These experiments showed that P211 exists in an equilibrium between single and double ring form. This opened the way to investigate the GroES-dependent and -independent refolding activity of a GroEL homologue as a single and as a double ring by simply varying the concentration of the chaperone. The chimeric protein P211T101R, which differs from P211 only by the mutation of threonine in position 101 to arginine, serves as a useful positive control, as it is always observed as a double ring on gels, and shows the same behavior with regard to ATPase activity at all the protein concentrations tested.

According to the model (10), described above, the double ring structure of GroEL and allosteric communication between the rings are essential for GroEL-ES-mediated protein folding. The model is strongly supported by the finding that single ring GroEL mutant SR1 can bind polypeptide, GroES, and nucleotide, and can hydrolyze ATP and allow polypeptides to fold in the cavity, but it cannot release the GroES and the peptide and is therefore unable to work as a chaperone (10). The results presented here show that P211 can, at high P211 concentrations, mediate refolding of mMDH in the presence of GroES and MgATP with similar rate and yield to wild type GroEL. At lower P211 concentration it loses this ability. But P211 is able to bind denatured mMDH at any concentration and to abolish any spontaneous refolding of mMDH in the absence of GroES and ATP. These findings support the model for GroEL-dependent protein folding described above. At high concentration the P211 forms double rings with the capacity for allosteric ring-ring interaction. Therefore ATP binding to the trans ring can trigger the release of GroES and mMDH from the cis ring. At low protein concentrations P211 exists predominantly in the single ring form. The single rings can bind mMDH and GroES but they cannot release them, and P211 thus cannot act as a chaperone. These results are in good agreement with the findings by Weber et al. (22) and Wang et al. (38), who demonstrated that the tetradecameric structure of GroEL is necessary for the refolding of the strictly GroES-dependent substrate proteins mMDH, rhodanese, and citrate synthase and that neither the single ring mutant SR1 nor the mini-chaperones could assist in refolding these proteins under stringent conditions.

A different result is seen with a GroES-independent substrate. GroEL can support LDH refolding in the absence of GroES (22, 36, 30). P211 can also support the folding of LDH at any P211 concentration, presumably because no GroES release is required. This indicates that the GroES-independent refolding mechanism is somewhat different and that at least for LDH refolding the double ring structure is not necessary. One can speculate that this could be due to a relatively weak binding affinity of the chaperone to LDH and that the function of the chaperone is not so much to provide an Anfinsen cage-like environment where the polypeptide can fold, but more to lower the concentration of free denatured protein to temporarily minimize the risk of aggregation.

It has been shown (22) that neither the SR1 single ring mutant nor the mini-chaperones can compensate in vivo for absence of GroEL. The finding that in vivo the level of P211 needed by the cells is higher than the level of GroEL or of the P211T101R mutant, which both have a stable double ring structure, strongly implies that the double ring structure is also essential for GroEL to act effectively in vivo. It is of course not possible to unambiguously determine the oligomeric state of any protein in the cell. However, given that P211 and P211T101R are so similar in sequence but show distinct differences in their allosteric properties at low protein concentrations, it seems reasonable to suppose that the different levels of the two proteins required for in vivo function is a consequence of their distinct allosteric behavior, and that P211 is unable to function in its single ring form in vivo. This is as predicted from the fact that GroES is also required for viability (1), which implies that at least some of the essential GroEL substrates are GroES dependent, and thus that the double ring structure will be required for in vivo function. This is the first clear demonstration that a chaperone can show a concentration-dependent shift in its properties that correlates with its behavior both in vitro and in vivo.

Acknowledgments—We are grateful to Tony Clarke, Neil Ranson, Steve Burston, and Neil Kadd (University of Bristol) for help with refolding assays and provision of pure GroES protein. We thank Roger George for providing the data for the Cpn60-1 protein and also A. Horovitz for generously hosting us in Israel.

REFERENCES

1. Fayet, O., Ziegelhoffer, T., and Georgopoulos, C. (1989) J. Bacteriol. 171, 1379–1385

3 E. J. Wallington and P. A. Lund, unpublished data.
A Double Ring Is Needed for GroEL-ES-mediated Folding

20357

2. Bukau, B., and Horwich, A. L. (1998) Cell 92, 351–366
3. Horovitz, A. (1998) Curr. Opin. Struct. Biol. 8, 93–100
4. Richardson, A., Landry, S. J., and Georgopoulos, C. (1998) Trends Biochem. Sci. 23, 138–143
5. Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A-L., and Sigler, P. B. (1994) Nature 371, 578–586
6. Boisvert, D. C., Wang, J., Otwinowski, Z., Horwich, A-L., and Sigler, P. B. (1996) Nat. Struct. Biol. 3, 170–177
7. Xu, Z., Horwich, A-L., and Sigler, P. B. (1997) Nature 388, 741–750
8. Roseman, A. M., Chen, S., White, H., Braig, K., and Saibil, H. R. (1996) Cell 87, 241–247
9. White, H. E., Chen, S., Roseman, A. M., Yifrach, O., Horovitz, A., and Saibil, H. R. (1997) Nat. Struct. Biol. 4, 690–694
10. Yifrach, O., and Horovitz, A. (1994) J. Mol. Biol. 243, 397–401
11. Yifrach, O., and Horovitz, A. (1995) Biochemistry 34, 5303–5308
12. Weissman, J. S., Hohl, C. M., Kovalenko, O., Kashi, Y., Chen, S., Braig, K., Saibil, H. R., Fenton, W. A., and Horwich, A. L. (1995) Cell 83, 577–587
13. Weissman, J. S., Rye, H. S., Fenton, W. A., Beechem, J. M., Xu, Z., Sigler, P. B., and Horwich, A. L. (1997) Nature 388, 792–798
14. Zahn, R., Buckle, A. M., Perrett, S., Johnson, C. M., Corrales, F. J., Golik, R., and Persh, A. R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5981–5986
15. Ben-Zvi, A. P., Chatellier, J., Fersht, A. R., and Goloubinoff, P. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15275–15280
16. Weber, F., Keppel, F., Georgopoulos, C., Hayer-Hartl, M. K., and Hartl, F. U. (1998) Nat. Struct. Biol. 5, 977–985
17. Nielsen, K. L., and Cowan, N. J. (1998) Molecular Cell 2, 93–94
18. Jones, S., Wallington E. J., George, R., and Lund, P. A. (1996) J. Mol. Biol. 262, 789–809
19. Yifrach, O., and Horovitz, A. (1995) Biochemistry 34, 5303–5308
20. Weissman, J. S., Rye, H. S., Burston, S. G., Atkinson, T., Holbrook, J. J., and Clarke, A. R. (1994) FEBS Lett. 344, 129–135
21. Staniforth, R. A., Cortes, A., Burstos, S. G., Atkinson, T., Holbrook, J. J., and Clarke, A. R. (1994) EMBO J. 15, 1397–1403
22. Staniforth, R. A., Cortes, A., Burstos, S. G., Atkinson, T., Holbrook, J. J., and Clarke, A. R. (1994) FEBS Lett. 344, 129–135
23. Miller, A. D., Maglhaes, K., Albanese, G., Kleinjan, D. A., and Smith, C. (1993) Biochem. J. 291, 159–164
24. Zettlmeisel, G., Rudolph, R., and Jaenicke, R. (1979) Eur. J. Biochem. 100, 593–598
25. Badcoe, L. G., Smith, C. J., Wood, S., Halsall, D. J., Holbrook, J. J., and Clarke, A. R. (1993) Biochemistry 32, 9195–9200
26. Wang, J. D., Michelitch, M. D., and Weissman, J. S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12163–121688
27. Rye, H. S., Burston, S. G., Fenton, W. A., Beechem, J. M., Xu, Z., Sigler, P. B., and Horwich, A. L. (1997) Nature 388, 792–798
28. Clarke, A. R., Waldman, A. D. B., Munro, I., and Holbrook, J. J. (1985) Biochem. Biophys. Acta 829, 375–379
29. Horovitz, A., Rochkareva, E. S., Kovalenko, O., and Girshovich, A. S. (1993) J. Mol. Biol. 231, 58–64
30. Ranson, N. A., Duster, N. J., Burston, S. G., and Clarke, A. R. (1995) J. Mol. Biol. 250, 581–586
31. Clarke, A. R., Waldman, A. D. B., Munro, I., and Holbrook, J. J. (1995) J. Biol. Chem. 270, 1121–1130
32. Stanisens, P., Opsomer, C., McKeown, Y. M., Kramer, W., Zabeau, M., and Fritz, H.-J. (1989) Nucleic Acids Res. 17, 4441–4454
33. Staniforth, R. A., Cortes, A., Burstos, S. G., Atkinson, T., Holbrook, J. J., and Clarke, A. R. (1994) EMBO J. 15, 1609–1614
34. Zettlmeisel, G., Rudolph, R., and Jaenicke, R. (1979) Eur. J. Biochem. 100, 593–598
35. Staniforth, R. A., Cortes, A., Burstos, S. G., Atkinson, T., Holbrook, J. J., and Clarke, A. R. (1994) FEBS Lett. 344, 129–135
36. Miller, A. D., Maghlaou, K., Albanese, G., Kleinjan, D. A., and Smith, C. (1993) Biochem. J. 291, 159–164
37. Badcoe, L. G., Smith, C. J., Wood, S., Halsall, D. J., Holbrook, J. J., and Clarke, A. R. (1993) Biochemistry 32, 9195–9200
38. Wang, J. D., Michelitch, M. D., and Weissman, J. S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12163–121688