doi:10.1016/j.jmb.2009.11.074 J. Mol. Biol. (2010) 396, 1271–1283

JMB

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

Characterisation of a GroEL Single-Ring Mutant that Supports Growth of *Escherichia coli* and Has GroES-Dependent ATPase Activity

Eszter Kovács[1,2], Zhe Sun[1,3], Han Liu[1,4], David J. Scott[5], Andreas I. Karsisiotis[1,6], Anthony R. Clarke[7], Steven G. Burston[7] and Peter A. Lund[1]*

[1]*School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK*

[2]*SOLVO Biotechnology, Gyár u. 2; Budaörs H-2040, Hungary*

[3]*Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, UK*

[4]*Physiological Laboratory, University of Liverpool, Liverpool L69 3BX, UK*

[5]*National Centre for Macromolecular Hydrodynamics, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Leicestershire LE12 5RD, UK*

[6]*Centre de Biophysique Moléculaire, UPR 4301 CNRS, 45071 Orléans cedex 2, France*

[7]*Department of Biochemistry, University of Bristol, School of Medical Sciences, Bristol BS8 1TD, UK*

Received 20 August 2009; received in revised form 26 November 2009; accepted 30 November 2009
Available online 16 December 2009

Edited by M. Gottesman

Binding and folding of substrate proteins by the molecular chaperone GroEL alternates between its two seven-membered rings in an ATP-regulated manner. The association of ATP and GroES to a polypeptide-bound ring of GroEL encapsulates the folding proteins in the central cavity of that ring (cis ring) and allows it to fold in a protected environment where the risk of aggregation is reduced. ATP hydrolysis in the cis ring changes the potentials within the system such that ATP binding to the opposite (trans) ring triggers the release of all ligands from the cis ring of GroEL through a complex network of allosteric communication between the rings. Inter-ring allosteric communication thus appears indispensable for the function of GroEL, and an engineered single-ring version (SR1) cannot substitute for GroEL *in vivo*. We describe here the isolation and characterisation of an active single-ring form of the GroEL protein (SR-A92T), which has an exceptionally low ATPase activity that is strongly stimulated by the addition of GroES. Dissection of the kinetic pathway of the ATP-induced structural changes in this active single ring can be explained by the fact that the mutation effectively blocks progression through the full allosteric pathway of the GroEL reaction cycle, thus trapping an early allosteric intermediate. Addition of GroES is able to overcome this block by binding this intermediate and pulling the allosteric pathway to completion via mass action, explaining how bacterial cells expressing this protein as their only chaperonin are viable.

Keywords: chaperonin; GroEL; single ring; allostery

*Corresponding author. E-mail address: p.a.lund@bham.ac.uk.

Abbreviations used: wtGroEL, wild-type GroEL; EDTA, ethylenediaminetetraacetic acid; CV, column volume.

Introduction

GroEL, the chaperonin of *Escherichia coli*, is a remarkable example of a complex, ATP-driven molecular machine. It is an essential protein *in vivo*[1] due to the fact that a number of essential cellular proteins are unable to fold to their active forms in its absence.[2] Its chaperone activity requires the presence of the co-chaperonin GroES, whose presence is also essential *in vivo*.[1] Although essential for the folding of only a relatively small number of proteins, it is able to interact with and assist the refolding of a significant proportion of the *E. coli* proteome.[2,3]

GroEL is a homo-oligomeric complex of 14 subunits arranged as two heptameric rings stacked back-to-back, with each ring surrounding a central cavity having an approximate volume of 85,000 Å^3.[4] Each GroEL subunit is composed of three domains: an equatorial domain that contains inter-ring contacts and most of the intra-ring contacts as well as the ATP binding site, an apical domain that contains the binding site for unfolded protein substrate and GroES, and a highly flexible intermediate domain that links the apical and equatorial domains. GroES is a smaller, dome-shaped homo-heptamer that is able to bind to one end of the GroEL cylinder in the presence of ATP or ADP.[5–7]

Unfolded protein initially binds in a compact state via exposed hydrophobic residues[8–11] to hydrophobic regions on the apical domains of the GroEL ring opposite (trans) to the one to which GroES is associated (cis). The association of ATP to the trans ring causes those subunits to proceed through a series of rapid ($t_{1/2}$ ~25–30 ms) conformational changes during which the trans-bound polypeptide substrate may be stretched out into a less compact conformation.[12,13] These conformational changes are also communicated across the inter-ring interface to force the ejection of GroES and protein substrate, whether folded or not, from the cis ring.[14–16] Weak binding of GroES to the trans ring now occurs before further rapid conformational changes in the trans ring displace the protein substrate into the enlarged central cavity[17–20] and tightens the interaction between GroES and GroEL over 1000-fold.[21] At this point the trans ring has become the new cis ring and *vice versa*. The protein substrate now has the opportunity to fold in isolation in the new cis cavity without the risk of aggregation. The rate of folding in the central cavity is sometimes enhanced compared to that seen in the bulk solvent. This is due to the fact that the nature of the folding energy landscape is changed within the GroEL central cavity.[22] Whether this is simply due to the elimination of the possibility of forming transient low-order aggregates[23,24] or whether it is due to folding in a confined space where the accessible volume has been reduced[25–28] remains controversial.[29] Once ATP has been hydrolysed to ADP in the cis ring the structure remains unchanged;[30] however, the energetics of the complex changes such that association with GroES is now weakened.[19,31] This relatively slow hydrolysis step provides a "timer" for the lifetime of the enclosed central cavity ($t_{1/2}$ ~6 s) and completes a "half-cycle".[14,19,32] The binding of protein substrate and ATP to the trans ring starts a fresh half-cycle. The reaction cycle continues in this manner with each ring alternating between being the cis folding chamber and the trans ring.

The GroEL reaction cycle requires highly coordinated structural dynamics to ensure that the chaperonin proceeds through its reaction cycle in the correct order and that efficient binding and release of substrate polypeptide and GroES are achieved.[33–35] The GroEL architecture has evolved, therefore, to transmit allosteric information between the subunits in each individual heptameric ring and also between the rings themselves across the ring–ring interface. This is reflected by the fact that ATP binds to each heptameric ring of GroEL with positive cooperativity; however, there is distinct asymmetry between the two rings such that the association of one GroEL ring with ATP weakens the affinity of the opposite ring for ATP, that is, negative cooperativity.[16,36,37]

The requirement for allosteric signalling between the two rings for completion of the cycle was experimentally demonstrated by the construction of a mutant form of GroEL, called SR1, which forms only single rings *in vitro*.[37] Protein substrates bound to SR1 can fold to their active form but remain trapped in the cavity beneath the GroES cap due to the lack of an allosteric signal from the absent trans ring forcing GroES and protein substrate release. As predicted by this model, mutants of GroEL with weakened ring–ring interactions can fold the GroES-dependent substrate mMDH when in a double-ring form, but not in a single-ring form, whereas the GroES-independent substrate LDH can be folded in both cases.[38] However, several examples of active single-ring chaperonins have been described, which either occur naturally or have been produced by mutagenesis with or without selection.[39–42] In all cases studied, these single-ring mutants are able to act because the interaction between the mutated GroEL and GroES is weak relative to that of the wild-type, removing the need for a signal from the trans ring for discharge of GroES and release of the substrate protein from the cavity.

The ATPase activity of wild-type GroEL, in the absence of substrate polypeptide, is inhibited by about 66% by the binding of an equimolar amount of GroES.[43] This is due to a change in the rate-limiting step of the reaction cycle from inter-ring communication immediately prior to ATP hydrolysis (in the absence of GroES) to a conformational change in the trans ring and its communication to the cis ring (in the presence of GroES).[16,31] SR1 possesses an intrinsic ATPase activity that is almost completely inhibited (once a single turnover of ATP to ADP has occurred) by the binding of GroES, although treatments that weaken the binding can partially restore some ATPase activity.[19,37] Active single-ring mutants of chaperonins generally show much reduced inhibition of ATPase activity by

GroES, reflecting the weakened interaction between GroES and these mutant proteins.[39,41] In a series of saturation mutagenesis experiments on SR1 designed to select for single rings that were active and could replace GroEL *in vivo*, we isolated one particular mutant (SR-A92T) several times independently, which functioned well in *in vivo* complementation assays.[42] We show here that this mutant has almost no detectable ATPase activity unless GroES is also present. This mutant has clearly been disturbed in its ATP hydrolytic cycle, and the fact that this can be overcome by the addition of GroES suggests that the problem lies with the ATP-induced structural changes that take place in a GroEL ring and that are central to the allosteric mechanism of this protein complex. It was decided therefore to engineer a single tryptophan residue (W485) that has been used previously as an intrinsic fluorescent probe to follow ATP-induced structural changes in both GroEL[21,44] and SR1[45] into the SR-A92T mutant. These ATP-induced allosteric changes in both GroEL and SR1 follow the scheme:

$$T \leftrightarrow R_1 \leftrightarrow R_2 \leftrightarrow R_3 \leftrightarrow R_4$$

while a further intermediate between R_3 and R_4 (termed R_3*) is populated in the presence of GroES.[21] Analysis of the kinetic pathway of ATP-induced allostery in SR-A92T provides an insight into the role of this residue in allosteric communication within GroEL.

Results

Initial identification and characterisation of the SR-A92T mutant

In an attempt to isolate as many possible mutants of SR1 as possible that were able to function as single rings, our original mutagenesis[42] was repeated several times, varying the mutagen used (hydroxylamine or error-prone PCR) and the stringency of the selection (either at 37 °C or at 42 °C). A mutant that was isolated independently at least four times contained a substitution of alanine by threonine at position 92. Because this was the most frequently isolated of any of the apparently active single-ring mutants in these experiments, it was selected for more detailed characterisation. This mutant is hereinafter called SR-A92T.

By deleting the chromosomal copy of the *groEL* gene, using P1 transduction from the strain AI90/pBAD50 as described,[46] it was confirmed that SR-A92T could function as the sole chaperonin in the cell. Plating of strains in which SR-A92T was the only expressed form of *groEL* showed that it was able to support growth up to 42 °C, although it formed colonies smaller than those of the wild-type control at high temperatures. A plasmid expressing SR-A92T was introduced into the strain SF103, which contains a temperature-sensitive *groEL* allele and which cannot plate the bacteriophage T4 or T5. Expression of SR-A92T in these strains restored its ability to grow at 42 °C and to plate bacteriophage T4 and T5 with an efficiency of plating of 100%.[42] SR-A92T is thus able to complement for loss of GroEL *in vivo* at normal growth temperatures, although it shows slightly reduced function under heat shock conditions. This shows that SR-A92T must be fully competent at folding proteins *in vivo*, which for several other active single-ring proteins was also confirmed by *in vitro* analysis.[42]

To determine whether other amino-acid substitutions at position 92 were equally competent at rescuing the function of SR1, the alanine at this position was mutated to all the remaining 18 amino-acids, and their ability to support growth of MGM100 on glucose at 37 °C was compared both with and without IPTG induction. The results showed that none grew as well as SR-A92T in the absence of IPTG, although SR-A92S was nearly as good, and several were slightly better than SR-A92T when induced to high levels (Table 1). Several mutants with major changes in side-chain character rescued the glucose-sensitive phenotype of MGM100 well when their expression was strongly induced, showing that there is significant structural capacity for variation without complete loss of function at this position. To see how well these results correlated with the natural variation at this position, chaperonin protein sequences in GenBank were used for comparison. Position 92 is one residue downstream from the highly conserved GDGTTT phosphate-binding loop motif and is very highly conserved in all chaperonins. A comparison of chaperonins from all three domains showed that the residue at this position is an alanine in nearly all type I chaperonins, including those from chloroplasts and mitochondria. Out of 782 bacterial GroEL homologues, in all cases where the GDGTTT motif is conserved (769), only one occurrence of a T was

Table 1. Effects of different substitutions in SR1 at position 92

Residue at position 92 in SR1	Growth at 37 °C in MGM100 on glucose−IPTG	Growth at 37 °C in MGM100 on glucose+IPTG
A	No growth	No growth
T	++++	+++
S	+++	++++
C	++	++++
V	+	++++
N	—	++++
M	—	++++
R	—	+++
Q	—	+++
Y	—	+++
L	—	+++
H	—	++
Wild-type GroEL	++++	++++

Growth was assessed by measuring the relative efficiency of plating of MGM100 (expressing each of the mutated forms of SR1 from the ptrc promoter in ptrc99A) at 37 °C on L-agar containing 0.2% glucose or to 0.2% arabinose. ++++, 100%; +++, 10%; ++, 1%; +, 0.1%. Substitutions not shown did not show any growth.

found at position 92, and the only other amino acids at this position were serine (six cases) and cysteine (seven cases). In type II chaperonins, however, a valine is quite common at this position. Thus, the amino acids that gave the best function in mutagenesis experiments are also the only other ones seen in this position in chaperonin proteins where the GDGTTT motif is intact. Only four examples were found in GenBank of chaperonins containing the sequence GDGTTTT: one in a bacterium (*Methylocella silvestris* BL2), one in an archaeon (*Halorubrum lacusprofundi*), one in a eukaryote (the zeta subunit of the type II TriC protein in the marine phytoplankton *Ostreococcus tauri*), and one in a bacteriophage (*Pseudomonas aeruginosa* phage EL).

SR-A92T is a single ring with a GroES-dependent ATPase activity

SR-A92T was purified to homogeneity from a strain where the chromosomal copy of *groEL* was deleted. Analytical centrifugation of the purified protein confirmed that it behaved hydrodynamically as a single-ring species. The presence of ATP, an ATP-regenerating system, or GroES did not change this result (see Supplementary Fig. 1).

The ATPase activity of 2 μM SR-A92T subunits was measured and found to be $1.87\times10^{-3}\pm5\times10^{-4}$ s^{-1} per subunit, 1.6% of the ATPase rate of wild-type GroEL (wtGroEL; 0.12 s^{-1} per subunit)[16] and ~1.3% that of SR1 (0.14 s^{-1} per subunit).[19,37,45,47] Remarkably, however, when GroES was added to the reaction in equimolar amounts, the ATPase rate increased 37-fold (to 0.069 ± 0.007 s^{-1} per subunit) to a level similar to that of wtGroEL in the presence of GroES (0.042 s^{-1} per subunit) (Fig. 1a). This contrasts with SR1 for which the presence of GroES reduces the rate of ATP hydrolysis by ~90% from 0.14 to 0.015 s^{-1},[45] due to the fact that it lacks the trans ring that normally provides an allosteric signal upon ATP binding and that leads to the ejection of GroES from the cis ring. Consequently, the SR1-$(ADP)_7$–GroES interaction is sufficiently tight that the very slow dissociation rate (τ ~1.1 min) of GroES from this complex limits the ATPase cycle.

SR-A92T has reduced affinity for GroES

SR1 is not viable in a GroEL-depleted background *in vivo*, as its ATPase cycle in the presence of GroES stalls after one round of ATP hydrolysis, due to the fact that there is no trans ring to bind ATP and transmit an allosteric signal.[37] Hence, GroES remains relatively tightly bound to the SR1-ADP_7 complex, thus trapping any folded substrate in the cavity. Since SR-A92T is able to suppress the nonfunctional phenotype of SR1 (in a wtGroEL-depleted background) it seems likely that the interaction between GroES and SR-A92T is sufficiently weakened to allow GroES, and hence polypeptide substrate, to dissociate more rapidly, thereby maintaining an encapsulation–ejection cycle. To determine the affinity of GroES for SR-A92T in the steady state, the GroES dependence of the steady-state rate of ATPase activity was determined (Fig. 1b). The resulting curve was fitted to a tight-ligand binding equation and yielded a binding stoichiometry of 1:1 and a K_d of 0.42 ± 0.02 μM, which is weaker than that seen for the binding of GroES to SR1 in steady state (K_d = 6–14 nM)[45] or wtGroEL in the presence of either ATP analogues (K_d = 12.5 nM)[48] or ADP (K_d = 0.3–3 nM).[49–51]

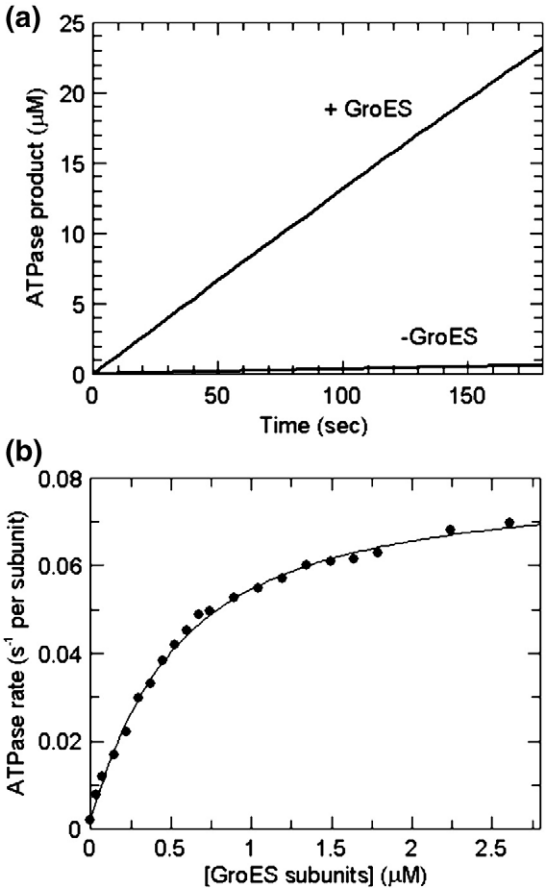

Fig. 1. (a) SR-A92T is a single ring with a GroES-dependent ATPase activity. SR-A92T (2 μM) subunits were mixed with 2 mM ATP in the absence or presence of 2 μM GroES and the rate of product evolution was recorded. The presence of GroES accelerates the very weak ATPase activity of SR-A92T by 37-fold from $1.87\times10^{-3}\pm5\times10^{-4}$ s^{-1} per subunit to 0.069 ± 0.007 s^{-1} per subunit. (b) SR-A92T has reduced affinity for GroES. SR-A92T (0.2 μM) subunits were used in a steady-state ATPase assay in the presence of increasing concentrations of GroES that results in an enhancement of ATPase activity. The resulting data were fitted to a tight-ligand binding equation with K_d of 0.42 ± 0.02 μM and the concentration of GroES binding sites (on SR-A92T) equal to 0.197 ± 0.01 μM (i.e., a 1:1 stoichiometry).

A92T increases the ATP hydrolytic activity of the double-ring wild-type GroEL

The A92T mutation was constructed in wild-type GroEL to assess its effects on the double-ring chaperonin. Expression of A92T-GroEL was able to

maintain cell viability in a GroEL-depleted background *in vivo*, demonstrating that it is still active as a chaperone. Measurements of the ATP hydrolytic rate showed that it was more active than wtGroEL in the absence of GroES (0.18 s^{-1} per active subunit compared to 0.12 s^{-1} for the wild-type chaperonin), and, while the addition of GroES inhibited this rate, its effect was not as great as that seen with wtGroEL (0.146 s^{-1} per active subunit, a 19% inhibition, compared to 0.041 s^{-1} for the wild-type, a 66% inhibition). Since the major energetic barrier to ATP hydrolysis in the double-ring chaperonin was identified to be an allosteric coupling between the two heptameric rings,[52] the increased rate of ATP hydrolyis in A92T-GroEL may be due to a partial uncoupling of this ring–ring allosteric communication.

The A92T mutation traps an allosteric intermediate

Since we can probe the allosteric transitions elicited by ATP binding using a single tryptophan probe, we used this approach to evaluate the effects of the A92T mutation on these rearrangements. In previous work,[44,45] single tryptophan versions (Y485W) of both double-ring GroEL and SR1 have been used to monitor the kinetics of these structural transitions by intrinsic fluorescence. Similar studies have used a different single tryptophan mutant, F44W, with similar observations.[47,53] The mutation Y485W (hereinafter called W485) was introduced into SR-A92T and the resulting double mutant was checked for its ability to support cell viability in a GroEL-depleted background and for its ATP binding and hydrolysis characteristics. In all of these, SR-A92T/W485 behaved exactly as did SR-A92T (data not shown). Upon rapidly mixing SR-A92T/W485 with ATP in the stopped flow, it was immediately apparent that SR-A92T/W485 does not proceed through the full complement of kinetic phases normally observed with SR1/W485 (or GroEL/W485). At a low concentration of ATP (75 μM), SR-A92T/W485 fluorescence rapidly increases upon mixing with the nucleotide (Fig. 2a) in a manner reminiscent of the initial kinetic phase seen with SR1/W485 (Fig. 2b) (and also GroEL/W485), albeit at a slower rate. However, two subsequent fluorescent-quenching phases are normally observed with SR1/W485 or GroEL/W485 followed by a slow fluorescence enhancement. This initial kinetic phase of fluorescence enhancement has not previously been quantitatively analysed in either SR1 or GroEL because it is usually too fast to accurately resolve at concentrations above ~100 μM and it is complicated by the subsequent quench. However, in the double-ring GroEL this phase has been assigned to either the first ATP-induced conformational change in one of the heptameric rings ($T{:}ATP_7 \rightarrow R_1{:}ATP_7$)[21,44] or the bimolecular association between ATP and GroEL.[53] Interestingly, when SR-A92T/W485 is mixed with ATP concentrations above 1 mM, a second, low-amplitude kinetic phase can be resolved in which the intrinsic fluorescence is

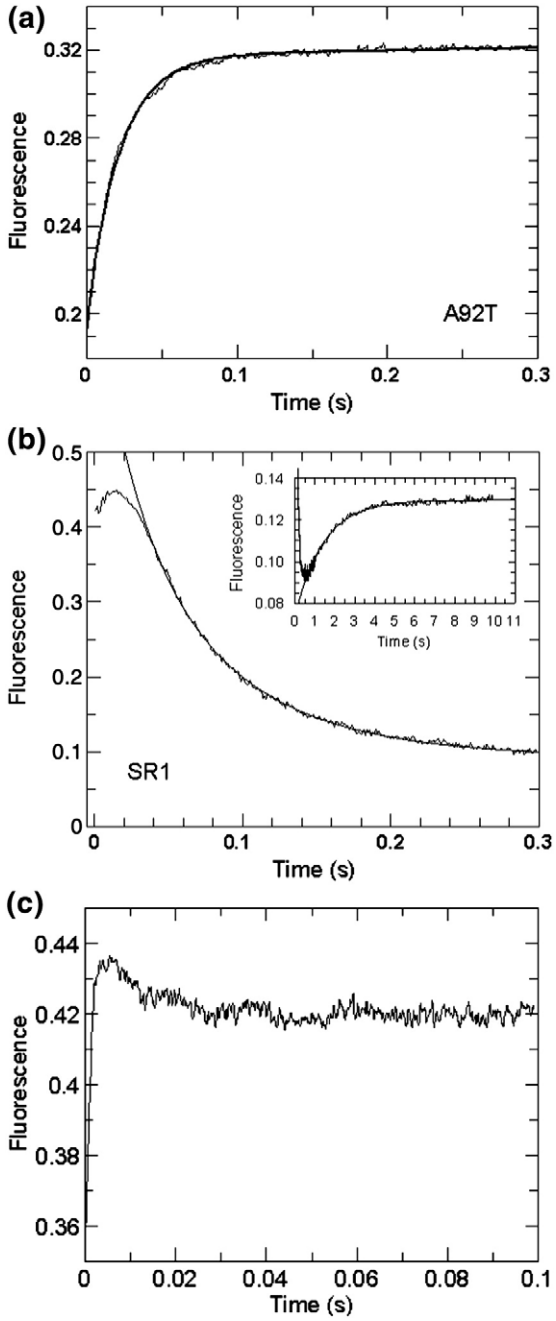

Fig. 2. The A92T mutation traps an allosteric intermediate. (a) SR-A92T/W485 heptamer (1 μM) was rapidly mixed with 75 μM ATP in a stopped-flow spectrofluorimeter and intrinsic fluorescence was monitored. The increase in fluorescence (T to R_1) fitted to a single exponential with rate constant = 46 ± 0.2 s^{-1}. No more kinetic phases could be observed at longer time scales. (b) SR1/W485 heptamer (1 μM) was rapidly mixed with 75 μM ATP and intrinsic tryptophan fluorescence was monitored. The increase in fluorescence is much more rapid than that observed with SR-A92T/W485 above. It also shows the presence of two fluorescence-quenching phases (R_1 to R_2 and R_2 to R_3 transitions) and the final slower fluorescence increase (R_3 to R_4 transition) (inset). (c) SR-A92T/W485 heptamer (1 μM) was rapidly mixed with 2 mM ATP in a stopped-flow spectrofluorimeter and intrinsic fluorescence was monitored. At this ATP concentration a small-amplitude quenching phase can now be observed.

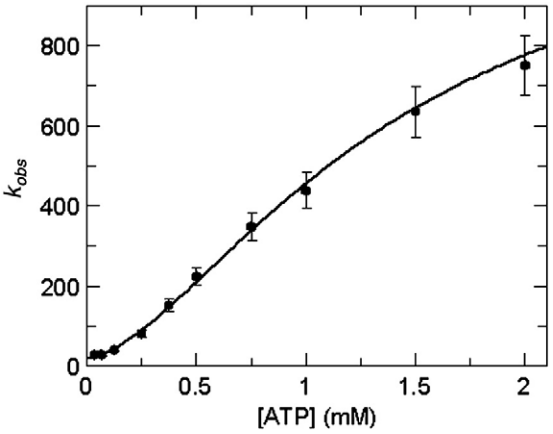

Fig. 3. The initial kinetic phase is not bimolecular. The observed rate constant for the initial fluorescence enhancement observed when 1 μM SR1/W485 heptamer was mixed with increasing concentrations of ATP shows a sigmoidal dependence, indicating that this phase is not reporting the initial bimolecular collision between ATP and SR1/W485 but a subsequent positively cooperative transition. The data were fitted to the Hill equation with maximal $k_{obs} = 1178 \pm 88\ s^{-1}$, $K_{1/2} = 1.39 \pm 0.14$ mM, and Hill constant = 1.61 ± 0.08, although the maximal k_{obs} is not well-defined by these data, since it becomes too fast to measure accurately.

quenched (see Fig. 2c at 2.5 mM ATP). This is similar to the first quenching phase observed with SR1. It therefore appears that SR-A92T does not proceed through its full complement of ATP-induced allosteric transitions but is stalled at a stage in which the first allosteric intermediate R_1 predominates with a minor population of R_2. No further progressions through to the intermediates R_3 or R_4 were evident.

The initial kinetic phase is not bimolecular

The equilibrium binding of ATP is positively cooperative.[43] However, kinetically this means that there must be a relatively weak binding of ATP to the T state governed by a bimolecular collision rate, which precedes the four kinetically distinct conformation rearrangements described previously for GroEL[44,53] and for the single-ring SR1,[47,49] which tighten the affinity for ATP. The initial bimolecular collision step will have a linear dependence on ATP concentration and so can be distinguished from the subsequent conformational rearrangement steps in that they must have either a hyperbolic (as in a two-step ligand-binding mechanism of collision followed by conformational change) or a sigmoid (when a more complex allosteric mechanism follows the initial bimolecular collision binding step) dependence on ATP concentration. To investigate whether the first observed kinetic phase (fluorescence increase) is due to a cooperative conformational change ($T{:}ATP_7 \rightarrow R_1{:}ATP_7$)[21,44] or to the bimolecular collision between ATP and the chaperonin,[54] we examined the dependence of the observed rate constant on ATP concentration (Fig. 3). These data reveal a sigmoidal dependence of k_{obs} on ATP concentration. Although k_{obs} is too fast to be accurately determined at high concentrations of ATP, the data can be fitted to the Hill equation with a maximal $k_{obs} = 1178 \pm 88\ s^{-1}$, $K_{1/2} = 1.39 \pm 0.14$ mM, and Hill constant = 1.61 ± 0.08. This fit is not fully accurate owing to the uncertainty in end point, but the observed cooperative behaviour shows that the initial, rapid enhancement is not due to the direct bimolecular collision of ATP and chaperonin, but reports a positively cooperative transition.

GroES binding relieves the kinetic block caused by the A92T mutation in SR1

The apparent lack of SR-A92T ATPase activity is overcome by the addition of GroES (Fig. 1a). Since the inhibition of the ATPase activity of SR-A92T appears to be due to the kinetic trapping of the pathway of ATP-induced structural rearrangements at the R_1 intermediate, we repeated the stopped-flow fluorescence experiments described above in the presence of GroES. Figure 4 shows the changes in the intrinsic fluorescence of SR-A92T upon rapidly mixing with ATP and GroES. The same experiment with SR1[45] shows three kinetic phases: a rapid increase in fluorescence followed by a fluorescence quench before a much slower final increase. Intriguingly, for the SR-A92T oligomer in the presence of GroES, we see the restoration of these optical phases. Since the pattern of fluorescence changes between the two proteins are the same and their rates are similar, this shows that (a) the presence of GroES ensures that SR-A92T proceeds through the full complement of ATP-induced conformational changes as seen with SR1, and (b) the presence of the A92T mutation has not qualitatively changed this kinetic pathway, but has affected the balance of the R_1-to-R_2 transition.

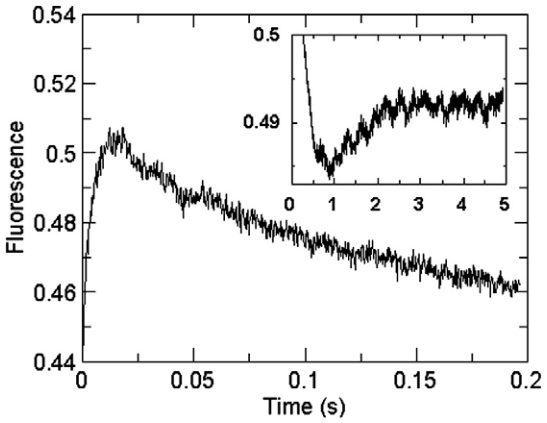

Fig. 4. GroES binding relieves the kinetic block caused by the A92T mutation in SR1. SR-A92T/W485 heptamer (1 μM) was rapidly mixed with 1 mM ATP and 2 μM GroES heptamer, and the intrinsic fluorescence change of SR-A92T/W485 was monitored. Three kinetic phases could be observed: a rapid increase in fluorescence and a slower quench phase before a much slower increase in fluorescence.

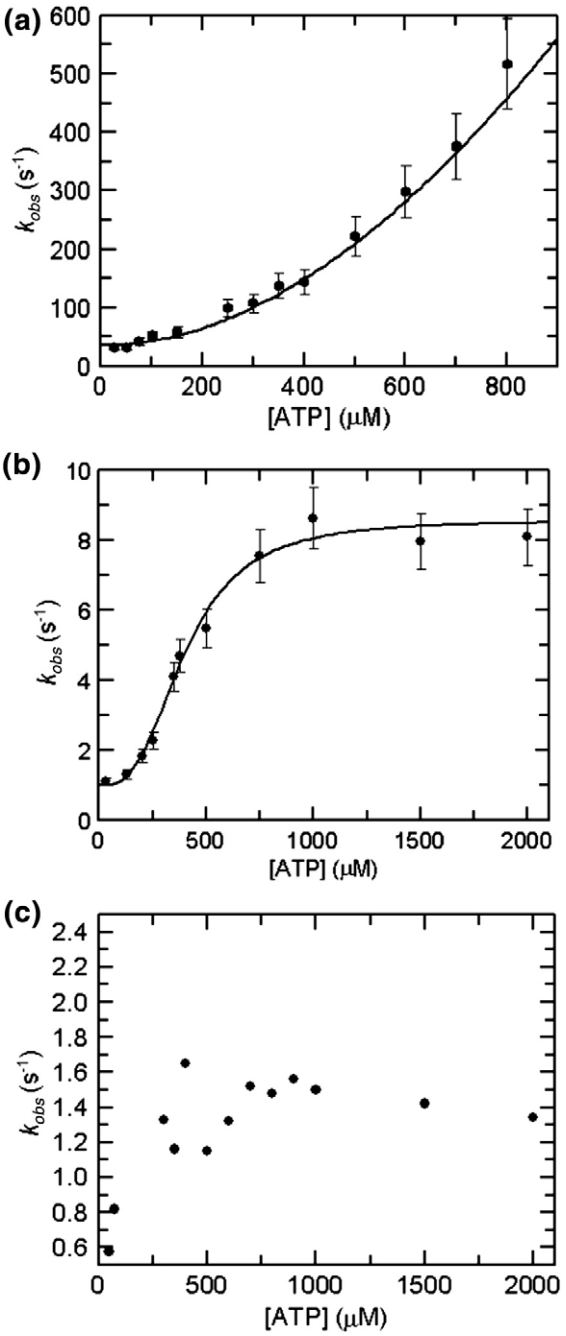

Fig. 5. ATP-dependence of the observed kinetic rate constants in the presence of GroES. The experiment shown in Fig. 4 was repeated in the presence of increasing concentrations of ATP and the observed rate constants for each kinetic phase were determined. (a) The ATP-dependence k_{obs} of the initial fluorescence enhancement phase. This was fitted to the Hill equation with parameters $k_{obs} = 8185 \pm 9816$ s^{-1}, $K_{1/2} = 5.03 \pm 3.73$ mM, y-intercept = 40.42 s^{-1}, and Hill constant = 2.04 ± 0.28, although the maximum value of k_{obs} is too large to be determined accurately. (b) The ATP-dependence of k_{obs} for the fluorescence quench phase. This was fitted to the Hill equation with parameters $k_{obs} = 8.6 \pm 0.46$ s^{-1}, $K_{1/2} = 0.4 \pm 0.03$ mM, and Hill constant = 2.88 ± 0.78. (c) The ATP-dependence of the final slow fluorescence enhancement phase shows that k_{obs} does not vary significantly with ATP concentration.

To investigate this further, the ATP concentration dependence of the observed rate constants of each phase was examined. The fast fluorescence increase (Fig. 5a) showed a similar ATP concentration dependence as the same phase in the absence of GroES (Fig. 3a). Although this phase becomes too fast at higher ATP concentrations to accurately determine k_{obs}, we have attempted to fit these data to the Hill equation with maximal $k_{obs} = 8185 \pm 9816$ s^{-1}, $K_{1/2} = 5.03 \pm 3.73$ mM, y-intercept = 40.42 s^{-1}, and Hill constant = 2.04 ± 0.28. These data are insufficient to accurately define the complete behaviour of this kinetic phase, but it is clear that this phase is too fast for stopped-flow measurements at high ATP concentrations, and the behaviour at low ATP concentrations suggests it is sigmoidal. This sigmoidal character again shows that this step is not the simple bimolecular binding of ATP but represents a subsequent conformational rearrangement.

The ATP dependence of the observed rate constant of the fluorescence quench phase that follows the initial, rapid fluorescence increase is shown in Fig. 5b. For SR-A92T, this phase fitted well to a single exponential at all concentrations of ATP rather than the double exponential seen for SR1. This is explained by the fact that in SR1 the R_1–R_2–R_3 series of transitions all have substantial amplitudes and GroES is not required to drag the R_1–R_2 transition. For the SR-A92T species, the R_1–R_2 transition lies heavily toward R_1 so there is only one discernable quench phase when GroES binds to the R_2 and R_3 forms and pulls the system through the sequence of rearrangements. The ATP dependence of k_{obs} for this phase was sigmoid in character and was optimally fitted to the Hill equation with parameters maximal $k_{obs} = 8.6 \pm 0.46$ s^{-1}, $K_{1/2} = 0.4 \pm 0.03$ mM, and Hill constant = 2.88 ± 0.78.

The final kinetic phase is a slower increase in fluorescence than has been observed previously with both wild-type GroEL (in the presence and absence of GroES) and SR1. As was the case with those proteins, k_{obs} is essentially independent of ATP concentration (Fig. 5c).

Discussion

GroEL and GroES constitute a molecular chaperone system within the cytosol of eubacteria that is essential for viability at all temperatures. Depletion of endogenous GroEL results in cell death that cannot be rescued by expression of the single-ring variant SR1, presumably because the SR1–GroES interaction is too tight in the absence of the allosteric signal from the trans ring.[19,37] However, random mutagenesis of SR1 identified a number of point mutations of SR1 that can rescue a GroEL-depleted strain of *E. coli*, including SR-A92T.[42] These mutants can completely replace wtGroEL in these cells. In an attempt to establish why SR-A92T no longer needs to function as a double-ring structure, we have examined the ATPase, GroES binding, and allosteric properties of this mutant single ring.

Unexpectedly, SR-A92T alone had almost no ATP hydrolytic activity when compared to SR1 (Fig. 1a), unlike the previous SR1 mutants that we studied.[42] However, in contrast to SR1, where GroES almost totally inhibits the steady-state ATPase, the co-chaperonin activated the ATP hydrolysis in SR-A92T to give a steady-state rate similar to that of wtGroEL in the presence of GroES. An essential part of the wtGroEL mechanism is the forcible ejection of GroES from the cis ring of GroEL once every half-cycle. This in turn allows substrate polypeptide to be released after it has had a chance to refold in the encapsulated environment of the cis cavity. The cis cavity in the wild-type chaperone decays with a $t_{1/2}$ of 16 s, a time that has evolved to be optimal for the efficient refolding of GroEL substrates.[55,56] If the lifetime of the cavity is too long, then GroEL will effectively retard the formation of active protein complexes. If the lifetime of the cavity is too short, then the proportion of the time that substrate proteins spend outside the cavity is greater and aggregation is more likely. The probable evolutionary pressure for the inter-ring, negative cooperativity is that in all circumstances, such a seesaw mechanism maintains an equal balance of rings in an acceptor state and an encapsulating state. The ATPase cycle of a single ring cannot proceed beyond a single turnover unless GroES has dissociated so we can deduce that the enclosed central cavity of SR-A92T decays with a half-time within the range that allows this mutant chaperonin to function sufficiently well *in vivo* under the conditions examined thus far.

Since the lifetime of the enclosed cavity underneath GroES is critical to the functioning of the chaperonin, we examined the affinity of GroES for SR-A92T by monitoring the increase in ATPase activity of SR-A92T as a function of GroES concentration (Fig. 1b). The affinity of SR-A92T for GroES in the steady state was 420 nM, which is almost 2 orders of magnitude weaker (~2–2.5 kcal/mol) than that determined for SR1 (K_d = 6–14 nM)[45] or wtGroEL–$(ADP)_7$ (K_d = 0.3–3 nM).[49–51] This is consistent with earlier studies on single-ring GroEL homologues and suggests that the dissociation rate of GroES is now sufficiently rapid to allow substrate polypeptides to cycle on and off the chaperonin in a manner that resembles that of the wild-type protein.

The reduction in affinity of SR-A92T for GroES could be explained as being either due to the mutation directly affecting the site of interaction between the single ring and GroES (extremely unlikely, since A92 is 35–40 Å from the GroES binding site) or due to the fact that mutating A92 has disturbed the communication of allostery throughout the structure, that is, by altering the relative stability of different conformational states. We investigated this latter possibility by making use of the well-established method of using changes in intrinsic fluorescence intensity of a single tryptophan at position 485 that has been engineered into the chaperonin. We have used this previously to determine the kinetic pathway of allosteric communication in GroEL[21,44] and SR1,[45] while a different single tryptophan mutant (F44W) has also been used with similar results.[47,53]

Upon ATP binding, wild-type GroEL and SR1 proceed through a complex series of shape changes T-R_1-R_2-R_3-R_4. We have previously interpreted these as ring opening (R_1), formation of the GroES receptor state (R_2), followed by the state that ejects protein substrate into the cavity (R_3). The formation of R_4 allows ATP hydrolysis.[21] In our current experiments, we find that SR-A92T proceeds through the T-R_1 rearrangement with only a trace of R_2 being formed. It would appear then that the mutation partially uncouples the allosteric effects of ATP binding by stabilizing R_1 with respect to the following conformational states and blocking the pathway.

We have identified previously that the first GroES-binding state of GroEL is R_2 and that subsequent structural rearrangements further tighten this association.[21] Hence, in the case of the SR-A92T species, this uncoupling can be overcome by the addition of GroES that binds to and therefore stabilizes the ensuing states (R_2 to R_4), thereby pulling the system through and restoring the ATPase activity. Having proceeded through the full complement of ATP-induced structural rearrangements, this ensures that the M-helix of the intermediate domain moves down toward the ATP binding site, bringing the important catalytic residue Asp398 into position for ATP hydrolysis to occur.[6,19] Once ATP hydrolysis has occurred, the subsequent SR-A92T–$(ADP)_7$–GroES complex has a weakened interaction between the SR-A92T and GroES, compared to that of either GroEL–$(ADP)_7$–GroES or SR1–$(ADP)_7$–GroES, and so the dissociation rate of GroES is sufficiently rapid to allow the release of substrate polypeptide from the central cavity at a rate that is optimal for normal cellular protein folding.[55]

The structural consequences of mutating Ala92 are not immediately apparent from inspection of the GroEL structure. While it is positioned close to the ATP binding site, occurring immediately after the phosphate-binding loop (threonines 89, 90, and 91) and at the N-terminal end of helix D that runs to the ring–ring interface (Fig. 6), alanine is typically a fairly benign amino acid being generally accepted into most positions in protein structure without causing significant perturbations. However, it is clear from image-reconstruction studies that helix D shifts between the GroEL–ATP and the GroEL–ATP–GroES conformations.[57] It can therefore be argued that replacing Ala92 with threonine (or other amino acids that were almost equally effective in reactivating SR1) may alter the relative energetic balance between these innate conformations of GroEL, favouring the former and inhibiting the transition to the state that binds GroES. In specific structural terms, disruption of the interactions between Ala92 at the top of helix D and Val77 and Ala78 at the end of helix C could potentially affect all of the helix C/D contact surface. Since the C-terminal end of helix D

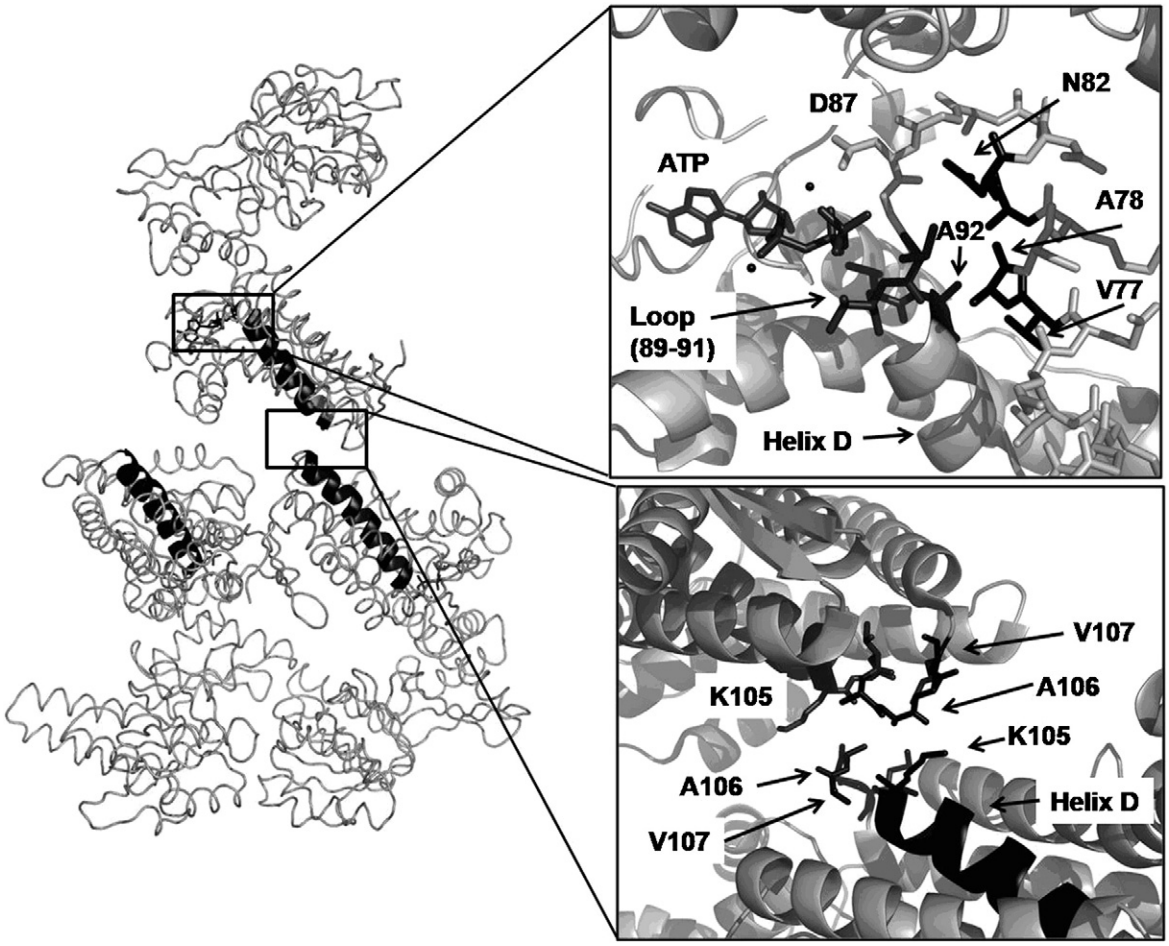

Fig. 6. The location of A92T in relation to a GroEL subunit and the ring–ring interface in wild-type GroEL. Left: One subunit of a GroEL heptameric ring is shown packed against two subunits from the opposite ring. The D-helix that runs from the ATP binding site to the ring–ring interface is shown in bold. Top panel, right: a zoom in on the subunit position of A92 showing its environment at the top of the D-helix next to the three threonine residues (89, 90, and 91) that form the phosphate-binding loop. Bottom panel, right: the contact region between D-helices of subunits in opposite rings. These figure were made using PyMol (DeLano Scientific) using Protein Data Bank coordinate set 1SX3.

makes contact with the same site on a subunit from the opposite ring in the wild-type GroEL molecule, and it is known that steric effects between the two heptameric rings are responsible for inter-ring allosteric communication, this subtle A92T mutation appears well placed to disrupt conformational coupling all the way from the GroES binding surface on the apical subunits through the nucleotide binding site and down to the equatorial interface between the rings. In addition, there is a peptide hydrogen bond between Ala92 and Gly88 at the opposite end of the phosphate-binding loop. This interaction (and others local to this) may result from the correct alignment of helices D and E. Substitution of Ala92 for Thr, and the resultant local structural movements to accommodate the threonine side chain, may disrupt the packing of helices D and E. In the wild-type protein, the tight packing of these helices, along with surrounding helices in the equatorial domain, may contribute to the equatorial domain acting as an effective stator as the apical domains move upward and rotate to bind GroES tightly. The energy for this is provided by the presence of ATP and the binding of GroES, as reported previously.[30] After ATP has hydrolysed to ADP, similar movements in the trans ring of the wild-type GroEL are responsible for transmission of the allosteric signal across the ring–ring interface to induce collapse of the apical domains and release of GroES and substrate polypeptide in the cis ring. Since this signal is absent in SR1, it is deficient in GroES release and cannot proceed through multiple cycles. However, if the A92T mutation in the single ring reported here results in an increased plasticity both in the equatorial domain and between helices D and E, then this will have the effect of reducing the free energy of the apical domain's opening and twisting in the presence of ATP. This in turn would lead to almost total absence of ATP hydrolysis, as observed for SR-A92T, as the allosterically induced structural rearrangements are required to bring the catalytic residue Asp398 into the active site. The tiny amount of ATP hydrolysis observed probably reflects the small amount of the R_2 kinetic intermediate reached when ATP is bound, assuming that this is the point in the pathway where the rotation

downward of the M helix (and Asp398) occurs. Upon addition of GroES, the co-chaperonin binds to the R_2 conformation[21] but results in the completion of the normal conformational change due to thermodynamic coupling with the GroES interaction. However, the net overall affinity of SR-A92T for GroES is approximately 2 orders of magnitude weaker when compared to the affinity for wild-type GroEL or SR1. The relative weakness of this interaction, caused by the increased plasticity in the region of the mutation, may allow the rapid collapsing of the apical domains to their apo conformation upon hydrolysis of ATP. This in turn permits GroES release (since ADP binding does not support the same structural movements as does ATP binding) and thus allows the mutant single ring to proceed through multiple cycles of binding and release in a manner similar to that of wild-type GroEL, although with reduced efficiency.

The finding that a single point mutation in SR1 can affect the ATP-induced allosteric pathway so dramatically is remarkable. In addition, the fact that ATP alone is now no longer sufficient to drive the full complement of structural changes but terminates at an intermediate stage of the kinetic allosteric pathway opens up the possibility of gaining direct structural information about such an intermediate using cryoelectron microscopy. Similar investigation of other mutations identified using the *in vivo* screen will determine whether these mutations effectively map out a part of the structural pathway involved in the transmission of allostery.

Materials and Methods

Bacterial strains and plasmids

The strain *E. coli* MGM100 is a derivative of MG1655 with the chromosomal *groE* promoter replaced by the pBAD promoter from the arabinose operon, which is strongly repressed on glucose.[58] The strain AI90/pBAD50 has the *groEL* gene replaced by a kanR cassette, with complementing *groEL* being provided from the plasmid pBAD50 under the control of the pBAD promoter.[46] The plasmid ptrcSR1 (AmpR) (a generous gift of A. Horwich and K. Furtak) is a derivative of the expression plasmid ptrc99A and contains the *groES* gene and the gene for the single-ring chaperonin SR1 under the control of the ptrc promoter.[37] This plasmid was used as the template for mutagenesis, and all complementation experiments and protein expression for purification were done using this plasmid or its mutated derivatives.

Growth conditions

All growth was on Luria broth or agar containing the appropriate antibiotics.

DNA manipulations

All site-directed mutagenesis was done using the Stratagene QuikChange® Site-Directed Mutagenesis Kit. Random mutagenesis was done using either hydroxylamine as described[42] or error-prone PCR. All mutations (both random and site directed) were checked by resequencing the entire *groE* operon, using the Applied Biosystems BigDye™ terminator method.

Protein purification

MGM100 or AI90 strains containing the plasmids expressing the Trp-GroEL, Trp-SR1, and Trp-A92TSR1 proteins were grown overnight in the presence of antibiotics and 0.2% L-arabinose and diluted into 1 liter of LB containing 0.2% glucose or 0.2% arabinose (in the case of ptrcSR1). Cultures were grown until A_{600} of 0.6 and induced by 0.1% IPTG for 5 h. Cells were harvested by centrifugation (8000***g***, 20 min), and the pellet was stored at −80 °C. The pellet was resuspended in the lysis buffer [2 ml per gram of cell pellet, 20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 500 μg/ml lysozyme, 50 μg/ml protease inhibitor cocktail (Sigma), 1 mM DDT, 20 μg/ml DNase, and 20 μg/ml RNase]. The suspension was stirred vigorously at 4 °C for 30 min, then at 37 °C for 30 min. Cells were lysed by sonication for a total time of 12 min with a 3-min pulse and 3 min on ice in each cycle (Cell Disruptor 200, Branson Ultrasonic Corp.). Cell debris was removed by centrifugation (30,000***g***, 30 min, 4 °C, repeated once). Then, glycerol was added to the supernatant to a final concentration of 20% and was included in all buffers throughout the purification to prevent the aggregation of the Trp mutant proteins. The supernatant was filtered with a 0.2-μm filter just before application to a DEAE Fast Flow column at 4 °C. The column was washed with 2 column volumes (CV) of buffer B [20 mM Tris–HCl (pH 7.5), 20% glycerol, 0.1 mM EDTA, and 0.05% sodium azide]. Proteins were eluted in a step gradient where 100% was buffer B containing 1 M NaCl: 0–20% 0.5 CV; on 20% 1.5 CV, 20–40% 2 CV (GroEL elutes here), 40–100% 0.5 CV. The column was regenerated on 100% 1 CV and equilibrated on 0% 2 CV. The fractions were analysed on 10% SDS–polyacrylamide gels, and fractions containing the target protein were mixed, concentrated by Vivaspin 20 (30,000 -kDa cutoff) concentrators (Vivascience), and dialysed against buffer C [50 mM Mes–NaOH (pH 6.0), 20% glycerol, 0.1 mM EDTA, and 0.05% sodium azide]. The protein was then applied to a Q Sepharose column. The column was washed with 2 CV of buffer C. Proteins were eluted in a step gradient where 100% was buffer C containing 1 M NaCl: 0–29% 1 CV, on 29% 1.5 CV, 29–50% 3 CV (GroEL elutes here), 50–100% 1 CV. The column was regenerated on 100% 1 CV, and equilibrated on 0% 2 CV. The fractions were analysed on 10% SDS–polyacrylamide gels, and fractions containing the target protein were mixed and concentrated by Vivaspin 20 (30,000 -kDa cutoff) concentrators (Vivascience).

The sample was then applied to a gel-filtration column (Sephacryl S-300) equilibrated and eluted with buffer B. Fractions were analysed and concentrated as before. Protein concentration was set to 2 mg/ml before removal of bound substrates with Affi-Gel Blue Gel (Bio-Rad). The protein was mixed with equal volumes of Affi-Gel Blue gel equilibrated in buffer B and incubated for 2 h at 4 °C with gentle shaking. This mixture was then centrifuged and the supernatant was filtered through a 0.2-μm filter to remove any remaining gel. For storage, 10 mM $MgCl_2$ and 10 mM KCl were added to the protein, which was stored at 4 °C.

To purify GroES, MGM100 cells containing the plasmid ptrcSR1 were grown, induced, harvested, and lysed as

described above. The supernatant was incubated (without adding any glycerol) at 80 °C for 30 min and centrifuged (30,000*g* for 30 min, twice) to remove the insoluble fraction. Glycerol was added to the buffers in the subsequent steps to improve protein stability. The supernatant was filtered and applied to a DEAE Fast Flow column. The column was washed with 1 CV buffer B, and GroES was eluted by application of a step gradient: 0–20% 0.5 CV, on 20% 1.5 CV (GroES elutes here and in the previous step), 20–40% 1 CV, 40–100% 0.5 CV. The column was regenerated on 100% 1 CV and equilibrated on 0% 2 CV. Fractions were collected, analysed by 10% SDS-PAGE, unified, and concentrated, and the buffer was changed for buffer C as in the case of GroEL, and SR1 purification. The protein was then applied to a Q Sepharose column. The column was washed with 2 CV buffer C and then eluted in a step gradient: 0–29% 1 CV, on 29% 1.5 CV (GroES elutes here and in the previous step), 29–50% 1 CV, 50–100% 1 CV. The column was regenerated on 100% 1 CV and equilibrated on 0% 2 CV. The fractions were analysed on SDS–polyacrylamide gels, and fractions containing the target protein were mixed, concentrated by Vivaspin 20 (30,000 -kDa cutoff) concentrators (Vivascience), and dialysed against buffer B. For storage, 10 mM $MgCl_2$ and 10 mM KCl were added to the protein, which was stored at 4 °C.

Chromatography was carried out on the Bio-Rad BioLogicLP system (DEAE, Q-Sepharose columns) and on the Amersham AKTA Explorer (gel filtration).

Steady-state ATPase assay

ATP hydrolysis was measured using the EnzCheck® Phosphate Assay Kit supplied by Molecular Probes, Inc.

Stopped-flow fluorescence measurements

All stopped-flow experiments were performed in buffer D [20 mM Tris–HCl (pH 7.5), 8% glycerol, 20 mM $MgCl_2$, 50 mM KCl, and 0.05% sodium azide] with an Applied Photophysics SX17MV stopped-flow spectrofluorometer. Tryptophan-containing mutants were excited by monochromated light at 295 nm and the resulting fluorescence was selected with a WG320 filter that cuts off all light below 320 nm. All reactions were performed at 25 °C and at least four to five transients were averaged for any data points.

Analytical methods

All stopped-flow initial data fittings were carried out with the stopped-flow software and refined with Grafit 3.0 (Erithacus software, 1994). All other data fittings were performed with Grafit 3.0. The data in Fig. 1b was fitted with the tight-ligand binding equation:[16]

$$v = \left(\frac{([SR] + [ES] + K_d) - \sqrt{([SR] + [ES] + K_d)^2 + 4[SR][ES]}}{2[SR]} \right) \times (v_{max} - v_{min}) + v_{min}$$

where v is the ATPase rate, v_{max} is the SR-A92T ATPase rate when saturated with GroES, v_{min} is the ATPase rate of SR-A92T in the absence of GroES, SR and ES represent SR-A92T and GroES, respectively, and K_d is the dissociation equilibrium constant for the interaction between SR-A92T and GroES.

Analytical ultracentrifugation

All sedimentation velocity experiments were performed in the XL-I ultracentrifuge using interference optics (Beckman Scientific Inc, Palo Alto, CA). Double-sector cells of 12 -mm optical path length were used with 390 μl of sample in each sector. Samples contained 0 or 2.16 μM purified SR-A92T, 0 or 6.48 μM GroES, 0 or 2 mM ATP, and a linked-enzyme ATP-regenerating system (PK/LDH) in buffer D. Cells were filled with buffer D containing ATP and phosphoenolpyruvate when it was necessary.

Acknowledgements

The authors thank Prof. Arthur Horwich and Dr. Krystyna Furtak for the kind gift of the SR1 plasmid. Additional mutagenesis experiments were done by John Solly, Kathryn Rolfe, and Cheryl Young. S.G.B., A.R.C., and P.A.L. acknowledge project funding from the BBSRC (UK). P.A.L. also acknowledges funding from the Darwin Trust of Edinburgh.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.11.074

References

1. Fayet, O., Ziegelhoffer, T. & Georgopoulos, C. (1989). The *groES* and *groEL* heat shock gene products of *Escherichia coli* are essential for bacterial growth at all temperatures. *J. Bacteriol.* **171**, 1379–1385.
2. Houry, W. A., Frishman, D., Eckerskorn, C., Lottspeich, F. & Hartl, F. U. (1999). Identification of in vivo substrates of the chaperonin GroEL. *Nature*, **402**, 147–154.
3. Chapman, E., Farr, G. W., Usaite, R., Furtak, K., Fenton, W. A., Chaudhuri, T. K. *et al.* (2006). Global aggregation of newly translated proteins in an *Escherichia coli* strain deficient of the chaperonin GroEL. *Proc. Natl Acad. Sci. USA*, **103**, 15800–15805.
4. Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L. & Sigler, P. B. (1994). The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. *Nature*, **371**, 578–586.
5. Hunt, J. F., Weaver, A. J., Landry, S. J., Gierasch, L. & Deisenhofer, J. (1996). The crystal structure of the GroES co-chaperonin at 2.8 Å resolution. *Nature*, **379**, 37–45.
6. Xu, Z., Horwich, A. L. & Sigler, P. B. (1997). The crystal structure of the asymmetric GroEL-GroES-(ADP)7 chaperonin complex. *Nature*, **388**, 741–750.
7. Saibil, H., Dong, Z., Wood, S. & auf der Mauer, A. (1991). Binding of chaperonins. *Nature*, **353**, 25–26.
8. Elad, N., Farr, G. W., Clare, D. K., Orlova, E. V.,

Horwich, A. L. & Saibil, H. R. (2007). Topologies of a substrate protein bound to the chaperonin GroEL. *Mol. Cell*, **26**, 415–426.
9. Fenton, W. A., Kashi, Y., Furtak, K. & Horwich, A. L. (1994). Residues in chaperonin GroEL required for polypeptide binding and release. *Nature*, **371**, 614–619.
10. Chen, L. & Sigler, P. B. (1999). The crystal structure of a GroEL/peptide complex: plasticity as a basis for substrate diversity. *Cell*, **99**, 757–768.
11. Falke, S., Tama, F., Brooks, C. L., 3rd, Gogol, E. P. & Fisher, M. T. (2005). The 13 angstroms structure of a chaperonin GroEL-protein substrate complex by cryo-electron microscopy. *J. Mol. Biol.* **348**, 219–230.
12. Lin, Z., Madan, D. & Rye, H. S. (2008). GroEL stimulates protein folding through forced unfolding. *Nat. Struct. Mol. Biol.* **15**, 303–311.
13. Lin, Z. & Rye, H. S. (2004). GroEL-mediated protein folding: making the impossible, possible. *Mol. Cell*, **16**, 23–34.
14. Todd, M. J., Viitanen, P. V. & Lorimer, G. H. (1994). Dynamics of the chaperonin ATPase cycle: implications for facilitated protein folding. *Science*, **265**, 659–666.
15. Weissman, J. S., Kashi, Y., Fenton, W. A. & Horwich, A. L. (1994). GroEL-mediated protein folding proceeds by multiple rounds of binding and release of nonnative forms. *Cell*, **78**, 693–702.
16. Burston, S. G., Ranson, N. A. & Clarke, A. R. (1995). The origins and consequences of asymmetry in the chaperonin reaction cycle. *J. Mol. Biol.* **249**, 138–152.
17. Weissman, J. S., Rye, H. S., Fenton, W. A., Beechem, J. M. & Horwich, A. L. (1996). Characterization of the active intermediate of a GroEL–GroES-mediated protein folding reaction. *Cell*, **84**, 481–490.
18. Mayhew, M., da Silva, A. C., Martin, J., Erdjument-Bromage, H., Tempst, P. & Hartl, F. U. (1996). Protein folding in the central cavity of the GroEL–GroES chaperonin complex. *Nature*, **379**, 420–426.
19. Rye, H. S., Burston, S. G., Fenton, W. A., Beechem, J. M., Xu, Z., Sigler, P. B. & Horwich, A. L. (1997). Distinct actions of cis and trans ATP within the double ring of the chaperonin GroEL. *Nature*, **388**, 792–798.
20. Clare, D. K., Bakkes, P. J., van Heerikhuizen, H., van der Vies, S. M. & Saibil, H. R. (2009). Chaperonin complex with a newly folded protein encapsulated in the folding chamber. *Nature*, **457**, 107–110.
21. Cliff, M. J., Limpkin, C., Cameron, A., Burston, S. G. & Clarke, A. R. (2006). Elucidation of steps in the capture of a protein substrate for efficient encapsulation by GroE. *J. Biol. Chem.* **281**, 21266–21275.
22. Brinker, A., Pfeifer, G., Kerner, M. J., Naylor, D. J., Hartl, F. U. & Hayer-Hartl, M. (2001). Dual function of protein confinement in chaperonin-assisted protein folding. *Cell*, **107**, 223–233.
23. Ranson, N. A., Dunster, N. J., Burston, S. G. & Clarke, A. R. (1995). Chaperonins can catalyse the reversal of early aggregation steps when a protein misfolds. *J. Mol. Biol.* **250**, 581–586.
24. Apetri, A. C. & Horwich, A. L. (2008). Chaperonin chamber accelerates protein folding through passive action of preventing aggregation. *Proc. Natl Acad. Sci. USA*, **105**, 17351–17355.
25. Klimov, D. K., Newfield, D. & Thirumalai, D. (2002). Simulations of beta-hairpin folding confined to spherical pores using distributed computing. *Proc. Natl Acad. Sci. USA*, **99**, 8019–8024.
26. Cheung, M. S. & Thirumalai, D. (2007). Effects of crowding and confinement on the structures of the transition state ensemble in proteins. *J. Phys. Chem. B*, **111**, 8250–8257.
27. Tang, Y. C., Chang, H. C., Chakraborty, K., Hartl, F. U. & Hayer-Hartl, M. (2008). Essential role of the chaperonin folding compartment in vivo. *EMBO J.* **27**, 1458–1468.
28. Tang, Y. C., Chang, H. C., Roeben, A., Wischnewski, D., Wischnewski, N., Kerner, M. J. *et al.* (2006). Structural features of the GroEL-GroES nano-cage required for rapid folding of encapsulated protein. *Cell*, **125**, 903–914.
29. Horwich, A. L., Apetri, A. C. & Fenton, W. A. (2009). The GroEL/GroES cis cavity as a passive anti-aggregation device. *FEBS Lett.* **583**, 2654–2662.
30. Chaudhry, C., Farr, G. W., Todd, M. J., Rye, H. S., Brunger, A. T., Adams, P. D. *et al.* (2003). Role of the gamma-phosphate of ATP in triggering protein folding by GroEL–GroES: function, structure and energetics. *EMBO J.* **22**, 4877–4887.
31. Rye, H. S., Roseman, A. M., Chen, S., Furtak, K., Fenton, W. A., Saibil, H. R. & Horwich, A. L. (1999). GroEL-GroES cycling: ATP and nonnative polypeptide direct alternation of folding-active rings. *Cell*, **97**, 325–338.
32. Roseman, A. M., Chen, S., White, H., Braig, K. & Saibil, H. R. (1996). The chaperonin ATPase cycle: mechanism of allosteric switching and movements of substrate-binding domains in GroEL. *Cell*, **87**, 241–251.
33. Saibil, H. R., Horwich, A. L. & Fenton, W. A. (2001). Allostery and protein substrate conformational change during GroEL/GroES-mediated protein folding. *Adv. Protein Chem.* **59**, 45–72.
34. Papo, N., Kipnis, Y., Haran, G. & Horovitz, A. (2008). Concerted release of substrate domains from GroEL by ATP is demonstrated with FRET. *J. Mol. Biol.* **380**, 717–725.
35. Yifrach, O. & Horovitz, A. (1994). Two lines of allosteric communication in the oligomeric chaperonin GroEL are revealed by the single mutation Arg196→Ala. *J. Mol. Biol.* **243**, 397–401.
36. Yifrach, O. & Horovitz, A. (1995). Nested cooperativity in the ATPase activity of the oligomeric chaperonin GroEL. *Biochemistry*, **34**, 5303–5308.
37. Weissman, J. S., Hohl, C. M., Kovalenko, O., Kashi, Y., Chen, S., Braig, K. *et al.* (1995). Mechanism of GroEL action: productive release of polypeptide from a sequestered position under GroES. *Cell*, **83**, 577–587.
38. Erbse, A., Yifrach, O., Jones, S. & Lund, P. A. (1999). Chaperone activity of a chimeric GroEL protein that can exist in a single or double ring form. *J. Biol. Chem.* **274**, 20351–20357.
39. Viitanen, P. V., Lorimer, G. H., Seetharam, R., Gupta, R. S., Oppenheim, J., Thomas, J. O. & Cowan, N. J. (1992). Mammalian mitochondrial chaperonin 60 functions as a single toroidal ring. *J. Biol. Chem.* **267**, 695–698.
40. Nielsen, K. L. & Cowan, N. J. (1998). A single ring is sufficient for productive chaperonin-mediated folding in vivo. *Mol. Cell*, **2**, 93–99.
41. Chatellier, J., Hill, F., Foster, N. W., Goloubinoff, P. & Fersht, A. R. (2000). From minichaperone to GroEL 3: properties of an active single-ring mutant of GroEL. *J. Mol. Biol.* **304**, 897–910.
42. Sun, Z., Scott, D. J. & Lund, P. A. (2003). Isolation and characterisation of mutants of GroEL that are fully functional as single rings. *J. Mol. Biol.* **332**, 715–728.

43. Gray, T. E. & Fersht, A. R. (1991). Cooperativity in ATP hydrolysis by GroEL is increased by GroES. *FEBS Lett.* **292**, 254–258.
44. Cliff, M. J., Kad, N. M., Hay, N., Lund, P. A., Webb, M. R., Burston, S. G. & Clarke, A. R. (1999). A kinetic analysis of the nucleotide-induced allosteric transitions of GroEL. *J. Mol. Biol.* **293**, 667–684.
45. Poso, D., Clarke, A. R. & Burston, S. G. (2004). A kinetic analysis of the nucleotide-induced allosteric transitions in a single-ring mutant of GroEL. *J. Mol. Biol.* **338**, 969–977.
46. Ivic, A., Olden, D., Wallington, E. J. & Lund, P. A. (1997). Deletion of *Escherichia coli* groEL is complemented by a *Rhizobium leguminosarum* groEL homologue at 37°C but not at 43°C. *Gene*, **194**, 1–8.
47. Amir, A. & Horovitz, A. (2004). Kinetic analysis of ATP-dependent inter-ring communication in GroEL. *J. Mol. Biol.* **338**, 979–988.
48. Behlke, J., Ristau, O. & Schonfeld, H. J. (1997). Nucleotide-dependent complex formation between the *Escherichia coli* chaperonins GroEL and GroES studied under equilibrium conditions. *Biochemistry*, **36**, 5149–5156.
49. Jackson, G. S., Staniforth, R. A., Halsall, D. J., Atkinson, T., Holbrook, J. J., Clarke, A. R. & Burston, S. G. (1993). Binding and hydrolysis of nucleotides in the chaperonin catalytic cycle: implications for the mechanism of assisted protein folding. *Biochemistry*, **32**, 2554–2563.
50. Todd, M. J., Viitanen, P. V. & Lorimer, G. H. (1993). Hydrolysis of adenosine 5′-triphosphate by *Escherichia coli* GroEL: effects of GroES and potassium ion. *Biochemistry*, **32**, 8560–8567.
51. Kovalenko, O., Yifrach, O. & Horovitz, A. (1994). Residue lysine-34 in GroES modulates allosteric transitions in GroEL. *Biochemistry*, **33**, 14974–14978.
52. Poso, D., Clarke, A. R. & Burston, S. G. (2004). Identification of a major inter-ring coupling step in the GroEL reaction cycle. *J. Biol. Chem.* **279**, 38111–38117.
53. Yifrach, O. & Horovitz, A. (1998). Transient kinetic analysis of adenosine 5′-triphosphate binding-induced conformational changes in the allosteric chaperonin GroEL. *Biochemistry*, **37**, 7083–7088.
54. Inobe, T., Arai, M., Nakao, M., Ito, K., Kamagata, K., Makio, T. *et al.* (2003). Equilibrium and kinetics of the allosteric transition of GroEL studied by solution X-ray scattering and fluorescence spectroscopy. *J. Mol. Biol.* **327**, 183–191.
55. Wang, J. D., Herman, C., Tipton, K. A., Gross, C. A. & Weissman, J. S. (2002). Directed evolution of substrate-optimized GroEL/S chaperonins. *Cell*, **111**, 1027–1039.
56. Farr, G. W., Fenton, W. A. & Horwich, A. L. (2007). Perturbed ATPase activity and not "close confinement" of substrate in the cis cavity affects rates of folding by tail-multiplied GroEL. *Proc. Natl Acad. Sci. USA*, **104**, 5342–5347.
57. Ranson, N. A., Clare, D. K., Farr, G. W., Houldershaw, D., Horwich, A. L. & Saibil, H. R. (2006). Allosteric signaling of ATP hydrolysis in GroEL–GroES complexes. *Nat. Struct. Mol. Biol.* **13**, 147–152.
58. McLennan, N. & Masters, M. (1998). GroE is vital for cell-wall synthesis. *Nature*, **392**, 139.