

Folding with and without encapsulation by *cis*- and *trans*-only GroEL–GroES complexes

George W. Farr^{1,2}, Wayne A. Fenton²,
Tapan K. Chaudhuri^{1,2,3}, Daniel K. Clare⁴,
Helen R. Saibil⁴ and Arthur L. Horwich^{1,2,5}

¹Howard Hughes Medical Institute and ²Department of Genetics, Yale School of Medicine, Boyer Center, 295 Congress Avenue, New Haven, CT 06510, USA and ⁴Department of Crystallography, Birkbeck College, Malet Street, London WC1E 7HX, UK

³Present address: Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, Hauz Khas, New Delhi 110016, India

⁵Corresponding author
e-mail: horwich@csb.yale.edu

Although a *cis* mechanism of GroEL-mediated protein folding, occurring inside a hydrophilic chamber encapsulated by the co-chaperonin GroES, has been well documented, recently the GroEL–GroES-mediated folding of aconitase, a large protein (82 kDa) that could not be encapsulated, was described. This process required GroES binding to the ring opposite the polypeptide (*trans*) to drive release and productive folding. Here, we have evaluated this mechanism further using *trans*-only complexes in which GroES is closely tethered to one of the two GroEL rings, blocking polypeptide binding by that ring. *In vitro*, *trans*-only folded aconitase with kinetics identical to GroEL–GroES. Surprisingly, *trans*-only also folded smaller GroEL–GroES-dependent substrates, Rubisco and malate dehydrogenase, but at rates slower than the *cis* reaction. Remarkably, *in vivo*, a plasmid encoding a *trans*-only complex rescued a GroEL-deficient strain, but the colony size was approximately one-tenth that produced by wild-type GroEL–GroES. We conclude that a *trans* mechanism, involving rounds of binding to an open ring and direct release into the bulk solution, can be generally productive although, where size permits, *cis* encapsulation supports more efficient folding.
Keywords: chaperonin/encapsulation/*in vivo* folding/protein folding

Introduction

Assistance in protein folding provided by GroEL, the double-ring bacterial chaperonin, is mediated by actions of binding non-native polypeptide in a central cavity through multiple hydrophobic contacts, followed by release of polypeptide directed by the binding of ATP and the small single-ring co-chaperonin, GroES (Grantcharova *et al.*, 2001; Thirumalai and Lorimer, 2001; Hartl and Hayer-Hartl, 2002). Release appears to occur in two ways with respect to the topology of the complex: one, termed *cis*, involves binding of ATP and GroES to the same ring as the

polypeptide; and the other, *trans*, involves binding of ATP/GroES to the opposite ring.

Release *in cis* has been characterized in detail for such GroEL–GroES-dependent substrate proteins as mitochondrial malate dehydrogenase (MDH; 33 kDa), rhodanese (33 kDa), and Rubisco from *Rhodospirillum rubrum* (51 kDa). For these substrates, small enough to fit inside the cavity of a GroEL ring capped by GroES, the binding of ATP and GroES to the same ring as the substrate triggers its rapid release from the GroEL cavity wall ($t_{1/2} < 1$ s), followed by commencement of productive folding inside the domed GroEL–GroES cavity (Mayhew *et al.*, 1996; Weissman *et al.*, 1996; Rye *et al.*, 1997). Several features of *cis* folding appear to favor its productivity. First, folding occurs in isolation. Non-native polypeptide in the *cis* space cannot interact with other species, preventing multimolecular aggregation from occurring (Rye *et al.*, 1997). Secondly, folding occurs in the presence of hydrophilic cavity walls, switched from their initial hydrophobic character by the rigid body movements that occur upon binding nucleotide and GroES (Xu *et al.*, 1997), which probably favors burial of exposed hydrophobic surfaces of the non-native protein and exposure of hydrophilic surfaces, both properties of the native state. Finally, folding proceeds in the setting of the confining walls of the cavity, which may limit the number of off-pathway states and favor productive routes to the native state, effectively changing the energy landscape for folding (e.g. Brinker *et al.*, 2001).

Inside *cis* ternary complexes of GroEL, GroES and substrate that are programmed to be long lived (e.g. with SR1, a single-ring version of GroEL, or with D398A, an ATP hydrolysis-defective mutant), virtually 100% of input substrate molecules reach the native active form, with kinetics of refolding resembling a wild-type reaction (Weissman *et al.*, 1996; Rye *et al.*, 1997). During the wild-type reaction, however, the GroEL complex cycles, with GroES, polypeptide and ATP hydrolysis products released from GroEL every 10–15 s (Todd *et al.*, 1994; Weissman *et al.*, 1994; Smith and Fisher, 1995; Ranson *et al.*, 1997). Here, the two GroEL rings alternate as *cis* ternary folding chambers (Rye *et al.*, 1999), with only a fraction (~3–5%) of input non-native molecules reaching the native form in any given cycle.

Recently, a mechanism for productive folding involving release *in trans* has been identified involving aconitase, an 82 kDa substrate protein, whose large size prevented it from being encapsulated *in cis* by GroES (Chaudhuri *et al.*, 2001). This substrate came to attention because, when it was imported into yeast mitochondria genetically deficient for either Hsp60 or Hsp10, the homologs of GroEL and GroES, the protein was found in insoluble aggregates (Dubaquíe *et al.*, 1998). *In vitro*, both GroEL and GroES were found to be required to mediate productive refolding

of aconitase diluted from denaturant (Chaudhuri *et al.*, 2001). The requirement for GroES in this reaction occurred at the step of polypeptide release, where the binding of both ATP and GroES to the GroEL ring opposite that bound by aconitase was required in order to release the protein. A similar *trans*-driven release has also been reported for an 86 kDa fusion protein joining MBP with the α -subunit of human branched chain ketoacid dehydrogenase (Huang and Chuang, 1999). In contrast to folding in the *cis* cavity, the *trans* mechanism of folding probably involves attempts at productive folding after release into the bulk solution. As with the *cis* reaction, the *trans* mechanism involves multiple rounds of binding and release for full recovery of the input molecules in the native state (Chaudhuri *et al.*, 2001).

The utilization by aconitase of a *trans* mechanism for reaching its native state, both *in vivo* and *in vitro*, raises significant questions about this mechanism. Could it also support folding of other substrates, e.g. smaller ones such as Rubisco and MDH, that can be folded *in cis*? If so, what is the efficiency of *trans* relative to *cis* folding, i.e. what is the relative importance of encapsulation? To evaluate the *trans* reaction further and to allow a direct comparison with *cis* in the case of smaller proteins, we have constructed and studied *trans*-only versions of GroEL–GroES.

Results

Construction of chaperonin complexes with GroES covalently tethered to one of the two GroEL rings

We conjectured that a *trans*-only complex of GroEL–GroES could be produced by covalently tethering GroES to one of the two GroEL rings in such a way that GroES would block entry of polypeptide into that GroEL ring but still associate with and dissociate from the apical domains of the tethered GroEL ring during the nucleotide cycle (Figure 1A). In particular, in the part of the reaction cycle where there is no nucleotide present in the tethered GroEL ring (and ATP in the opposite ring, Figure 1A, top middle), the tethered GroES would not associate with the apical domains of the ring to which it was attached, but, if tethered closely enough, could sterically block entry of a substrate protein into the ring. Upon binding ATP in the tethered ring, the normal interaction of the tethered GroES with the nucleotide-mobilized apical domains of the tethered ring would now occur, forming an asymmetric GroEL–GroES complex (Figure 1A, bottom right). Such association in ATP has been shown to be required to drive release of polypeptide from the opposite open (*trans*) ring in the *trans* reaction (Huang and Chuang, 1999; Chaudhuri *et al.*, 2001). Subsequent ATP hydrolysis in the GroES/ATP-bound (tethered) ring to form an asymmetric GroEL–GroES-ADP complex (Figure 1A, bottom left) would then allow ATP and substrate protein to enter the opposite open (*trans*) ring (Figure 1A, left), in turn dissociating the tethered GroES (and ADP) from the apical domains of the tethered ring (Figure 1A, top middle). Notably, in the absence of free GroES, there would be no opportunity for encapsulation of polypeptide bound in the open, non-tethered ring, and thus the cycling tethered complex functions as *trans*-only.

We examined the model of the asymmetric GroEL–GroES-ADP₇ crystal structure (Xu *et al.*, 1997) to

determine a position for an appropriate linkage. Two residues at the outside aspect of the GroEL–GroES complex, lying 36 Å apart, were chosen: the C-terminal alanine (amino acid 97) of GroES and the side chain of GroEL residue 315 in the *cis* ring (Figure 1B). GroES Ala97 lies at the terminus of a β -strand that extends from inside to outside the subunit, pointing into the bulk solution. Residue Asn315 of GroEL lies at the outside aspect of the apical domain and previously has been substituted with cysteine in an otherwise cysteineless version of GroEL (GroEL 315C) without compromise of function (Rye *et al.*, 1999). To span the 36 Å between GroES 97 and GroEL 315C, a bipartite linkage was established (Figure 1B). At the GroES aspect, the primary sequence of the subunit was extended beyond Ala97 with a flexible Ser–Gly–Gly tripeptide repeated two or four times, followed by a cysteine residue, to yield a 24.5 or 31.5 Å extension, respectively. The GroES derivatives, termed GroES₁₀₄ and GroES₁₁₀, respectively, were both fully active with wild-type GroEL and ATP in promoting refolding of MDH in a control study. The remaining distance between the single C-terminal cysteines of the GroES derivatives and the cysteines of the GroEL 315C tetradecamer was bridged by reaction with the homobifunctional chemical cross-linker 1,8-*bis*-maleimido-triethyleneglycol [BM(PEO)₃], adding 14.7 Å (Figure 1B).

Cross-linking was carried out with a 2-fold molar excess of the GroES derivatives over GroEL 315C, in the presence of ADP to drive formation of asymmetric GroES–GroEL binary complexes. The GroEL-containing products of the cross-linking reactions were purified by anion exchange chromatography in the absence of nucleotide, allowing any non-cross-linked GroES molecules to dissociate from GroEL and separate from it. An aliquot of the GroEL-containing products was examined by SDS–PAGE (Figure 1C for products of the GroEL + GroES₁₀₄ reaction), and an adduct migrating substantially more slowly than GroEL was reproducibly detected. The amount of this species measured ~10% that of GroEL, suggesting that only one or perhaps two GroEL subunits had become cross-linked to a GroES subunit in each GroEL–GroES complex. An alternative possibility was that only ~5–10% of input GroEL complexes had become cross-linked to GroES, but with as many as seven linkages per GroEL–GroES complex. This explanation was excluded, however, by both biochemical and electron microscopy (EM) analyses.

Characterization of cross-linked GroEL–GroES complexes

The adduct from the GroEL–GroES₁₀₄ cross-linking reaction was subjected to sequential Edman degradation and, consistent with it being a GroEL–GroES cross-link product, both the N-terminal sequence of GroEL and that of GroES were detected (Figure 1C) in approximately equimolar amounts, consistent with this being a 1:1 cross-link.

The biochemical estimation of the percentage of GroEL molecules bearing a cross-linked GroES involved partial proteinase K digestion of the cross-linked complexes incubated in ADP. This treatment produces selective proteolytic removal of the flexible C-terminal tails from the subunits of open GroEL rings, but not from the

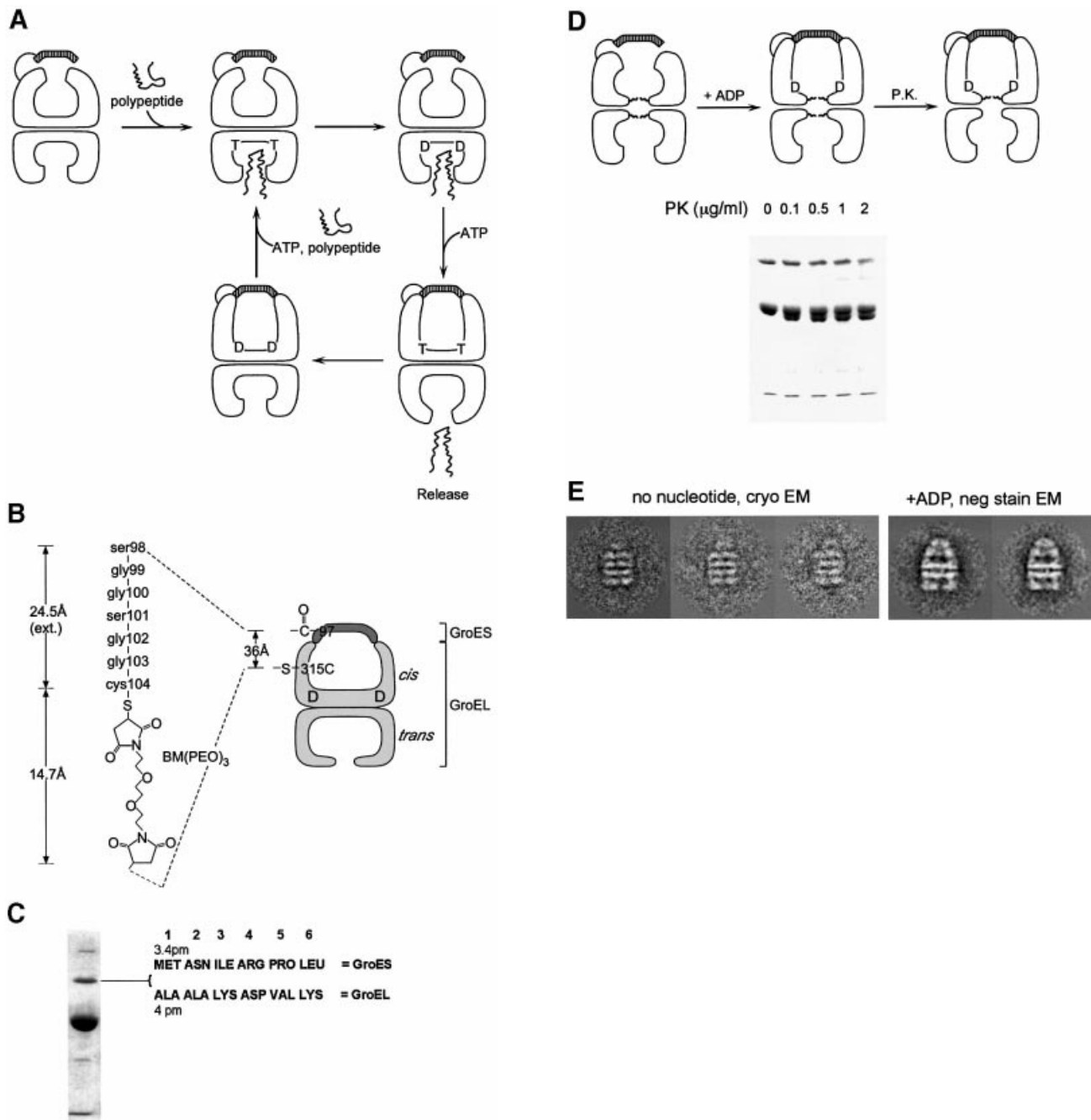


Fig. 1. Construction of *trans*-only GroEL–GroES complexes. (A) Functional cycle of a *trans*-only complex formed by closely tethering GroES to a GroEL ring. Polypeptide is sterically blocked from entering the tethered GroEL ring and can thus only bind and release *in trans* to GroES. In the absence of free GroES, no *cis* folding can occur. (B) Schematic showing the bipartite tether linking GroES to GroEL. GroES was extended at the coding sequence level by seven amino acids, and a homobifunctional maleimide cross-linker, BM(PEO)₃, was employed to link the C-terminal cysteine with GroEL apical Cys315. The predicted total length of the tether, 39.2 Å, is shown relative to the distance, 36 Å, between the normal C-terminus of GroES and GroEL315, determined from GroEL–GroES–(ADP)₇ (Xu *et al.*, 1997). (C) Products of the cross-linking reaction. SDS–PAGE analysis of anion exchange fractions containing GroEL species. The indicated species migrating more slowly than GroEL was subjected to N-terminal sequencing. Equivalent amounts of two N-termini were observed, corresponding to those of GroEL and GroES. Note that the N-terminal methionine of GroEL is normally removed *in vivo*, whereas that of GroES is not. (D) Most of the GroEL complexes from the cross-linking reaction bear one or two cross-links to a GroES complex. The purified products of the cross-linking reaction (1 μM) were incubated with 5 mM ADP to allow cross-linked GroES molecules to associate with the apical domains of the GroEL ring to which they were tethered (upper panel schematic). Partial proteinase K treatment was then carried out with varying amounts of enzyme as indicated, cleaving the flexible C-termini of open GroEL rings but not those of rings bound by GroES. Cleaved and uncleaved GroEL subunits were resolved by SDS–PAGE (bottom panel). (E) EM analyses of tethered GroEL–GroES₁₀₄ complexes. Selected averages of cryoEM side views in the absence of nucleotide after classification (left images); and averaged negative stain side views of complexes incubated with ADP (right images). In the absence of nucleotide, the tethered GroES can be seen as an additional weaker layer of density at the top of the molecule, but the GroEL itself is in the unliganded conformation. In the presence of ADP, the tethered GroES associates with the mobilized GroEL apical domains to form the typical GroEL–GroES complex, in which the GroES-bound ring of GroEL has undergone large domain rotations to form a physical association with GroES. The views shown contain ~10 raw images per class.

subunits of rings bound with nucleotide and GroES, leading to the proteolytic truncation of only 50% of the GroEL subunits in an asymmetric GroEL–GroES complex (Langer *et al.*, 1992). Here, addition of ADP alone should produce GroEL–GroES–ADP₇ asymmetric complexes whenever a GroEL complex contained a tethered GroES (see Figure 1D schematic). When the cross-linked pro-

ducts were treated in this way and analyzed by SDS–PAGE, we observed ~50% ‘clipped’ subunits and 50% ‘unclipped’ (Figure 1D), indicating that essentially all of the GroEL complexes were cross-linked to a GroES, i.e. that each had a GroES attached to one of the two GroEL rings. This implied then that there were only one or two covalent linkages between the GroEL and GroES rings.

The high percentage of GroEL molecules bearing a cross-linked GroES was established further by EM analyses of the cross-linked molecules, carried out in both the

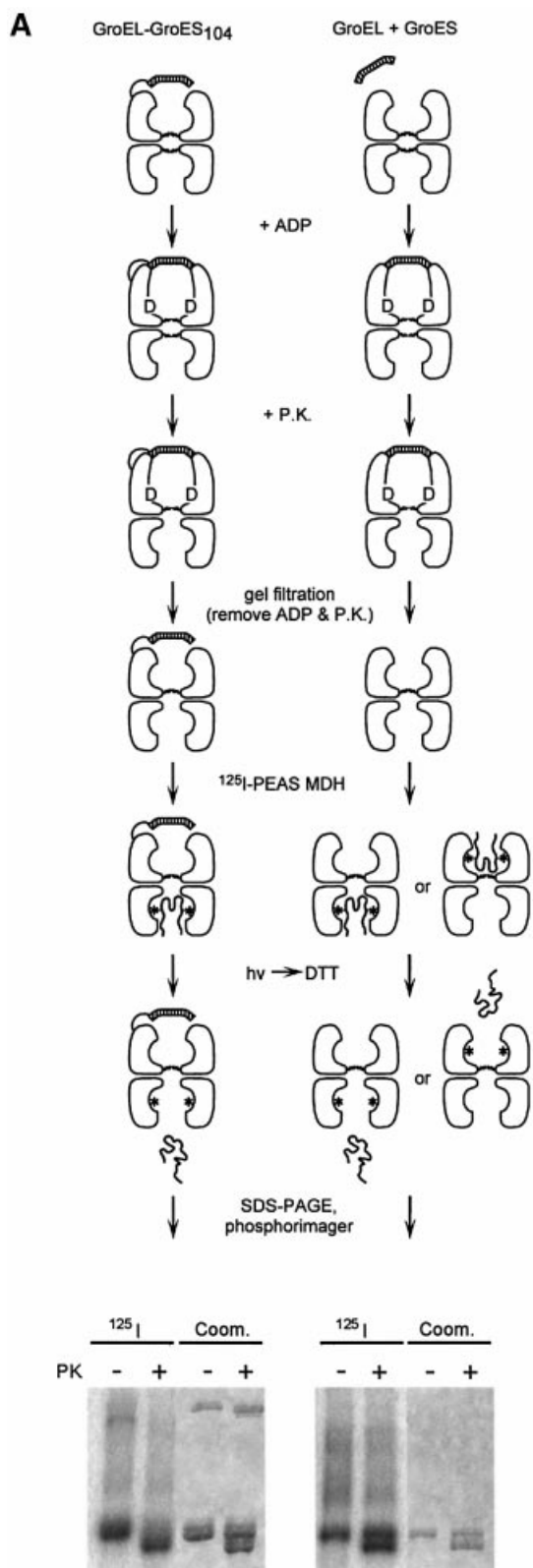


Fig. 2. Polypeptide binds only to the *trans* ring of GroEL–GroES₁₀₄. (A) Hit-and-run cross-linking experiment, illustrated schematically (top) and analyzed by SDS–PAGE (bottom). The non-tethered GroEL ring of GroEL–GroES₁₀₄ (left) or one ring of wild-type GroEL (right), both 1 μM, was ‘marked’ by partial proteinase K treatment (0.5 μg/ml), +, or not, –, in the presence of 5 mM ADP (GroEL–GroES₁₀₄) or 5 mM ADP and 2 μM GroES (GroEL) (first three panels). Gel filtration in the absence of nucleotide released ADP and GroES from GroEL, with GroES remaining tethered but unbound in the case of GroEL–GroES₁₀₄ (panel 4, left), while being completely removed in the case of wild-type GroEL (panel 4, right). A binary complex was formed between the marked chaperonins (1 μM) and unfolded MDH (0.2 μM), bearing a radiolabeled photoactivatable cross-linker, [¹²⁵I]PEAS, on one or more of its cysteine residues (panel 5). The complexes were photolyzed to cross-link to the proximate GroEL ring, and the cross-linker was released from the MDH by DTT (panel 6). (For clarity, polypeptide is drawn here as released from the chaperonin cavity, although it probably remains associated with the ring.) SDS–PAGE and phosphorimager analysis showed that only the marked ring was radiolabeled in the case of GroEL–GroES₁₀₄ (bottom left), indicating binding exclusively *in trans*. In contrast, both rings of wild-type GroEL were radiolabeled (bottom right), consistent with random binding. (B) Protease protection of MDH in binary complexes with *trans*-only. Binary complexes were formed between unfolded, radiolabeled MDH (0.5 μM) and either 1 μM GroEL plus 2 μM GroES (GroEL/ES), 1 μM GroEL–GroES₁₀₄ (EL-ES₁₀₄), or 1 μM GroEL–GroES₁₁₀ (EL-ES₁₁₀). Treatment of the complexes with 0.5 μg/ml proteinase K was carried out in the absence (+P.K.) or presence of 5 mM ADP (+ADP, P.K.). The reaction mixtures were separated by SDS–PAGE and radioactivity detected by phosphorimager. In each case, the –P.K. lane is the binary complex before proteinase K treatment. The diagrams below the experimental lanes indicate the interpretation of the data: wild-type GroEL–GroES complex protects about half of the input MDH because both *cis* and *trans* complexes form with about equal efficiency; GroEL–GroES₁₀₄ protects none of the MDH because only *trans* complexes form; GroEL–GroES₁₁₀ protects some MDH, but less than wild-type, because the longer tether permits some protected *cis* complex to form. Because of this last result, GroEL–GroES₁₁₀ was not studied further.

absence and presence of ADP (Figure 1E). A significant portion of GroEL molecules observable in side view exhibited extra density at one end of the molecule. In the absence of nucleotide, cryoEM analysis revealed a mass corresponding to GroES positioned in various attitudes at one end of the GroEL cylinder (class averages shown in Figure 1E, left panel). Because GroES was apparently linked to GroEL through only one or two cross-links, this would allow the variety of attitudes to be occupied, explaining why, even within a class average, the GroES was not as well resolved as GroEL. Yet in these class averages, GroES was positioned across the central axis, sitting over at least some portion, if not the entirety, of the mouth of the central cavity of the apposed GroEL ring. Notably, the apical domains of the apposed GroEL ring occupied a non-elevated position, identical to that of the opposite non-tethered ring, indicating that the tethered GroES did not produce elevation and association with the apical domains. In contrast, after addition of ADP to the cross-linked products, the majority of particles observable in side view exhibited the classic asymmetric morphology, with GroES physically associated with the elevated apical domains of the apposed GroEL ring (Figure 1E, right panel). These observations support that most of the GroEL complexes bear a tethered GroES (see also Supplementary data available at *The EMBO Journal* Online), that the topology of the tethered GroES should produce steric obstruction to polypeptide entry into the GroES-tethered GroEL ring and that the physical association of the tethered GroES with the apical domains of the attached GroEL ring is able to proceed in a normal manner in the presence of nucleotide.

The cross-linked GroEL–GroES₁₀₄ complex binds non-native MDH only in trans

To assess whether the cross-linked GroEL–GroES₁₀₄ could function as a *trans*-only complex in polypeptide binding, two types of study were carried out. In one, hit-and-run cross-linking was carried out much as in earlier studies (Weissman *et al.*, 1995), here attaching a radioiodinated heterobifunctional cross-linker, PEAS [N((2-pyridyldithio)ethyl)-4-azidosalicylamide], to cysteine residues of the substrate protein MDH unfolded in urea. The modified protein was then diluted into a mixture with GroEL–GroES₁₀₄ whose rings had already been ‘clipped’ proteolytically by partial proteinase K digestion in ADP (followed by removal of nucleotide by gel filtration; see Figure 2A schematic). The binary complex mixture was photolyzed and then reduced, transferring the cross-linker from the bound MDH to GroEL. The cross-linker-bearing (radiolabeled) GroEL subunits were then assessed by SDS–PAGE and phosphorimager analysis. Only clipped GroEL subunits, i.e. those originating from the non-tethered GroEL ring, were radiolabeled [Figure 2A, bottom left; compare + lanes Phosphorimager (¹²⁵I) with Coomassie-stained (Coom.)], indicating that MDH bound only to the open *trans* ring and not to the GroES-tethered GroEL ring.

In a second study using a protease protection assay, [³⁵S]methionine-radiolabeled MDH bound by GroEL–GroES₁₀₄ in the absence of nucleotide was unable to be protected from partial proteinase K digestion after addition of ADP (Figure 2B), indicating that MDH had not bound

to the GroES-tethered ring in the absence of nucleotide. In contrast, ~30% of input MDH was protected with wild-type GroEL and GroES (Figure 2B) (see additional text in the Supplementary data).

Trans-only GroEL–GroES₁₀₄ complex folds the trans substrate aconitase identically to wild-type GroES

To assess whether the *trans*-only complex could function to mediate folding of a substrate protein, we first tested the 82 kDa protein, aconitase, which can only be folded *in trans* by GroEL and GroES. Aconitase was diluted from acid into either a mixture with wild-type GroEL and GroES or one with the *trans*-only GroEL–GroES₁₀₄ complex, and ATP was added. Refolding of aconitase to its native form was measured at various times by assaying enzymatic activity. As shown in Figure 3, inset, the kinetics of refolding in the wild-type and *trans*-only reactions were identical, with nearly complete renaturation achieved by 3 min. Single turnover experiments for both chaperonin mixtures were carried out with ADP-aluminum fluoride (AlF₃), which mimics the step of ATP binding; both reactions achieved ~30% recovery, amounting to an increment of 15% over the spontaneous recovery of 15% in the absence of chaperonin. Thus, the *trans*-only complex appears to behave as efficiently and with the same characteristics as wild-type GroEL and GroES in mediating ATP-dependent refolding of a large substrate protein that can only be folded *in trans*.

Trans-only GroEL–GroES₁₀₄ complex refolds smaller substrates, but more slowly than wild-type GroEL–GroES

To test further the folding activity of the *trans*-only complex, we next examined its action on smaller substrate proteins that are folded by wild-type GroEL–GroES *in cis*. Subunits of Rubisco (51 kDa) and of mitochondrial MDH (33 kDa) stringently require the complete chaperonin system—GroEL, GroES and ATP—for refolding from denaturant. Would the *trans*-only complex have any activity in refolding these substrates, or could these proteins only be renatured in a *cis* cavity? The proteins were unfolded in acid/urea or guanidine-HCl, respectively, then diluted into mixtures containing *trans*-only or wild-type GroEL and GroES. ATP was added, and recovery of the native form was measured by enzyme activity assays at various times (Figure 4). We observed that, in the wild-type reaction, Rubisco was nearly fully renatured by 15 min, whereas with the *trans*-only complex, only ~20% of Rubisco activity was recovered by this time (Figure 4A). Recovery in the *trans*-only reaction reproducibly reached ~40% by 1 h. Overall, the rate of folding of Rubisco by wild-type GroEL–GroES was 4–6 times faster than by *trans*-only, and the extent of recovery from *trans*-only appeared to be reduced. In contrast, a single-ring version of *trans*-only (SR-X-ES) failed to refold Rubisco (Figure 4B), probably because it was unable to bind the non-native substrate. Likewise, as in earlier studies, in the absence of chaperonin or the presence of GroEL and ATP alone, no appreciable refolding of Rubisco was observed (Figure 4A).

The refolding of MDH was also compared between wild-type GroEL–GroES and the *trans*-only GroEL–GroES₁₀₄

complex (Figure 4C). Once again, a slower recovery of activity was observed for the *trans*-only complex, although here the extent of recovery approached that of wild-type GroEL–GroES by 1 h.

***Trans*-only folding of MDH and Rubisco requires cycles of release and rebinding**

The question arose as to whether the *trans*-mediated folding of these smaller substrates involves cycles of release into the bulk solution and rebinding, as occurs during rounds of *cis* folding of these proteins by wild-type GroEL–GroES, i.e. with release into solution occurring after each trial of folding in the *cis* cavity. To address this, the chaperonin trap molecule, D87K, was introduced into the *trans*-only reaction at various times after ATP addition (Figure 5). If non-native forms were being released at each round of the *trans* reaction cycle, then the D87K trap would bind (and not release) these species, preventing them from a further chance at rebinding and renaturation. In the case of MDH, this was observed when D87K was added at 0 and 8 min, at times when 0 or 40% of MDH molecules had already reached native form, respectively (Figure 5A). In the case of Rubisco, the trap was tested when added at initiation of the reaction, and here productive folding was completely blocked (Figure 5B). Thus, as had been indicated for the large substrate, aconitase (Chaudhuri *et al.*, 2001), *trans*-sided folding of smaller substrates also requires cycles of release and rebinding that probably comprise trial and error attempts at reaching the native state. Presumably the action of rebinding a non-native polypeptide molecule rescues it from irreversible misfolding, and cycles of such rebinding would ensue until any given substrate molecule reaches the native state.

A plasmid encoding a trans-only GroEL–GroES complex rescues a GroE-deficient strain

While folding of aconitase in mitochondria requires both Hsp60 and Hsp10 and is almost certainly mediated by a *trans* mechanism, and while, as just shown, several smaller GroEL–GroES-dependent substrates can be folded *in trans in vitro*, the issue remained as to whether *trans*-sided folding could be sufficient *in vivo* to support folding of the as yet unidentified essential cellular substrates of GroEL–GroES. In particular, could a *trans*-only version of GroEL provide sufficient function to rescue a GroEL–GroES-deficient cell? To address this, a second *trans*-only GroEL–GroES complex was programmed at the coding sequence level (Figure 6A). This construct, *transin vivo*, took advantage of an observation made during earlier 7-fold tandemization of the GroEL-coding sequence (programming a ring as a single polypeptide) (Farr *et al.*, 2000), namely that, when two GroEL-coding sequences are placed in tandem as a continuous open reading frame, they produce tetradecamers that are fully functional (G.W.Farr, H.R.Saibil, A.L.Horwich, unpublished). Because side-by-side pairs of GroEL subunits could not produce a seven-membered ring, we concluded that, for this so-called ‘dimer’, the two-component GroEL subunits must arrange into opposite rings. That is, the flexible C-terminal tail of the upstream GroEL subunit (termed U1) must extend across the equatorial plane to the opposite ring, connecting to the N-terminus of the downstream GroEL subunit (U2) (Figure 6B). We conjectured that all of the dimer subunits in a tetradecamer might be aligned in the same way, i.e. with all of the U1s forming one ring and the U2s forming the other. If so, then GroES subunits could be programmed at the coding sequence level to be fused to only one of the two rings by joining the C-terminus of U2, through a flexible tether, to the

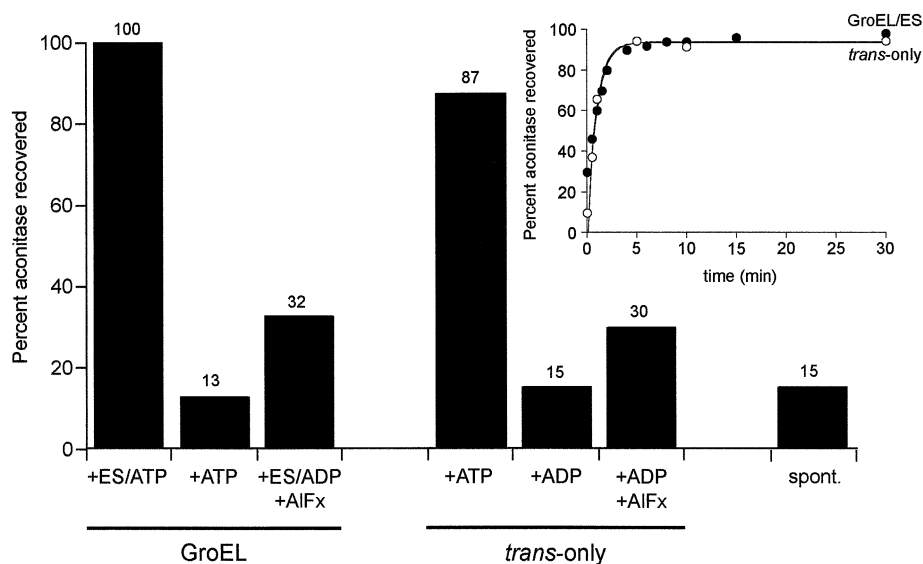


Fig. 3. The *trans* substrate aconitase is refolded by *trans*-only similarly to wild-type GroEL–GroES. Binary complexes were formed between unfolded aconitase, 1 μ M final concentration, and 1 μ M wild-type GroEL or GroEL–GroES₁₀₄ (*trans*-only). Refolding was initiated with the addition of 5 mM nucleotide, aluminum fluoride (AIF_x) and 2 μ M GroES (ES), as indicated beneath the lanes. The recovery of aconitase activity in the wild-type GroEL–GroES-ATP reaction was set to 100%; the lane labeled spont. is the recovery of aconitase activity in the absence of any chaperonin. The inset shows the time course of activity recovery in the GroEL–GroES-ATP (filled circles) and *trans*-only-ATP (open circles) reactions.

N-terminus of GroES. Although the C-terminal 25 residues of GroEL (here U2) are known to be flexible,

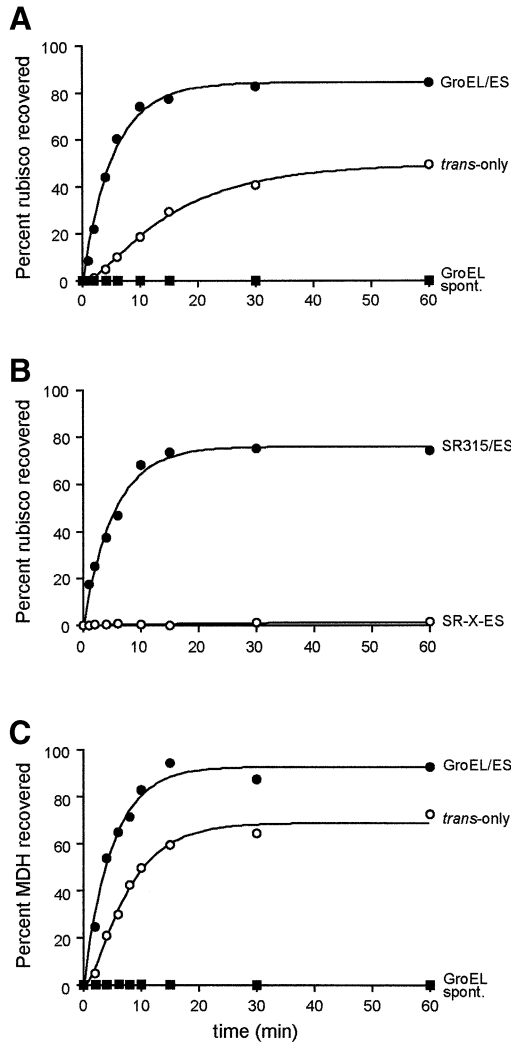


Fig. 4. *Trans*-only also refolds smaller substrate proteins that can fold *in cis*. **(A)** Time course of Rubisco refolding. Binary complexes were formed between 0.2 μM wild-type GroEL or GroEL–GroES₁₀₄ (*trans*-only) and unfolded Rubisco, 0.1 μM final concentration. ATP (5 mM) was added to both and 0.4 μM GroES to the wild-type complex, and Rubisco activity was determined in aliquots removed at the indicated times. Recovery is reported as the percentage of the starting Rubisco activity before unfolding. Wild-type GroEL + GroES + ATP (filled circles); GroEL–GroES₁₀₄ + ATP (open circles); GroEL + ATP alone or spontaneous refolding (spont.) (filled squares). **(B)** Time course of Rubisco refolding by single-ring versions of GroEL. Binary complexes were formed as in (A) between the single-ring version of GroEL315C (SR315) or SR315 cross-linked to GroES₁₀₄ similarly to GroEL–GroES₁₀₄ (SR-X-ES) and unfolded Rubisco. ATP and GroES were added to the SR315 complex, and ATP alone to SR-X-ES. At the indicated times, hexokinase and glucose were added to aliquots, and they were incubated for 30 min at 4°C to release GroES from the apical domains of the single ring (Rye *et al.*, 1997) before assaying Rubisco activity. As above, recovery is the percentage of the starting Rubisco activity. SR315 + GroES + ATP (filled circles); SR-X-ES + ATP (open circles). **(C)** Time course of MDH refolding. Binary complexes were formed between 2 μM wild-type GroEL or GroEL–GroES₁₀₄ (*trans*-only) and unfolded MDH, 1.2 μM final concentration. ATP (5 mM) and 4 μM GroES were added as in (A), and MDH activity was determined in aliquots removed at the indicated times. Recovery is the percentage of the starting MDH activity. GroEL + GroES + ATP (filled circles); GroEL–GroES₁₀₄ + ATP (open circles); GroEL + ATP alone or spontaneous refolding (spont.) (filled squares).

they localize mainly to the equatorial zone of the central cavity, as indicated by EM studies. Therefore, an additional flexible segment of 34 amino acids was added between U2 and GroES, allowing ~ 100 Å of tether to be present between the equatorial zone of the central cavity and the N-terminus of GroES as it is positioned in the GroEL–GroES–ADP₇ crystal structure (Figure 6B). The tether sequence was composed of repeating Ser–Gly–Gly tripeptides with a basic amino acid interspersed every tenth residue. The length of the tether seemed likely not to be as critical here as for GroEL–GroES₁₀₄ for attaining a steric block of polypeptide entry, because the seven tethers, putatively extending from each U2 subunit to each subunit of GroES, would themselves provide steric obstruction.

The *transin vivo* construct was overexpressed in *Escherichia coli* and purified through chromatographic steps identically to GroEL, although with somewhat reduced yield. In cryoEM examination of the purified protein in the absence of nucleotide, a mass corresponding to GroES was observed at one end of the GroEL cylinder

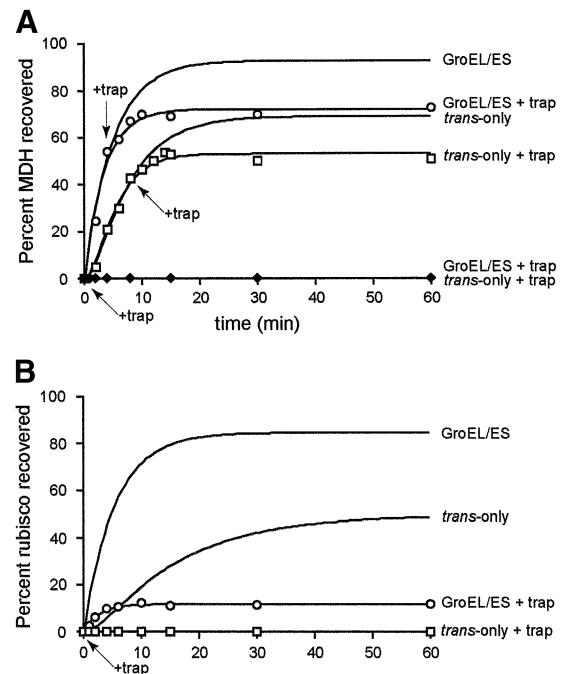


Fig. 5. Smaller substrates recycle during refolding by *trans*-only. **(A)** Time course of MDH refolding in the presence of a ‘trap’ mutant of GroEL. Binary complexes were formed as in Figure 4C, and the refolding reactions initiated as before, except that an 8-fold excess of D87K, a trap mutant of GroEL that binds unfolded polypeptide but does not release it, was added at 0 min (filled diamonds) (both GroEL/GroES + trap and *trans*-only + trap), 4 min (open circles) (GroEL/GroES + trap) or 8 min (open squares) (*trans*-only + trap), and MDH activity was determined as before. The GroEL–GroES and *trans*-only traces are reproduced from Figure 4C. Here, the addition of the trap to an ongoing folding reaction largely prevents further folding of any remaining non-native substrate polypeptide. **(B)** Time course of Rubisco refolding in the presence of trap. Binary complexes were formed as in Figure 4A, and a 20-fold excess of trap was added at the start of the reaction (open circles, GroEL/GroES + trap; open squares, *trans*-only + trap). Rubisco activity was determined as before. The GroEL–GroES and *trans*-only traces are reproduced from Figure 4A. The presence of the trap severely (GroEL–GroES) or completely (*trans*-only) inhibits the refolding reaction by preventing recycling of non-native polypeptide.

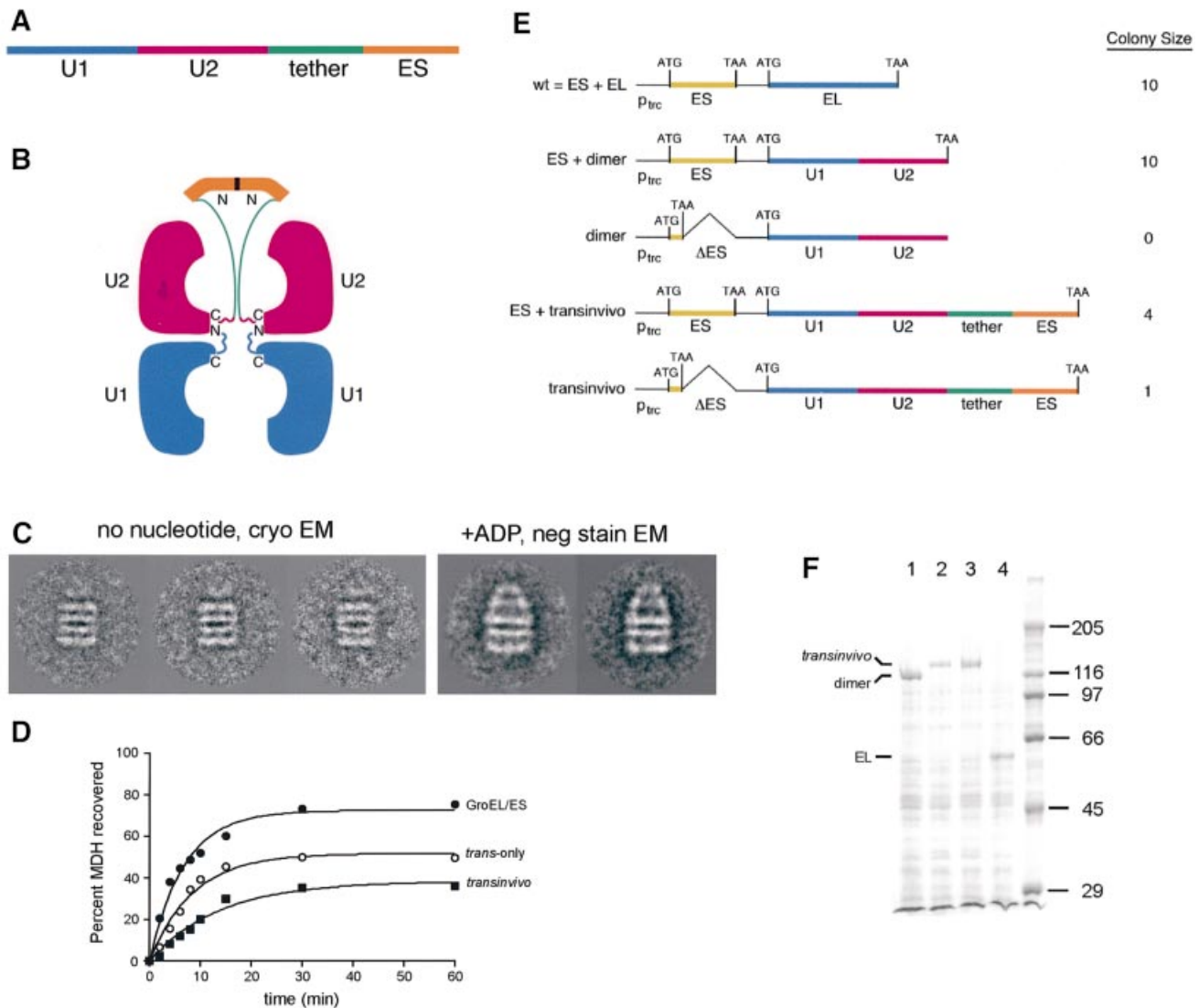


Fig. 6. Rescue of a *groE*-deficient *E. coli* strain by a *transin vivo* plasmid, encoding a *trans*-only GroEL–GroES complex. (A) Schematic of *transin vivo* coding sequence, in which two tandem GroEL-coding sequences, termed U1 and U2, are joined in turn to that of GroES via a segment encoding a flexible tether (see text). (B) Predicted topology of *transin vivo*, showing the collective of N-terminal GroEL units (U1s) forming the open non-tethered ring (blue), the collective of C-terminal GroEL units (U2s) forming the tethered ring (purple) and the flexible tethers (green) joining the flexible tails of U2s (purple wavy lines), respectively, to the N-termini of the subunits of GroES (orange). (C) Class averages of cryoEM side views of purified *transin vivo* complex in the absence of nucleotide (left), and class averages of negative stain side views of the complex in the presence of ADP (right). In the absence of nucleotide, the additional density at the top of GroEL is attributed to the tethered GroES, while GroEL itself remains in an unliganded conformation, resembling the *trans*-only GroEL–GroES₁₀₄ complex (Figure 1E). As before, in the presence of ADP, typical GroEL–GroES complexes are observed. The averages contain ~10 raw images per class. (D) Refolding of MDH *in vitro* mediated by purified *transin vivo*, compared with a wild-type reaction and with the *trans*-only complex. Binary complexes with MDH were prepared as described in Figure 4C, folding was initiated with either ATP (*transin vivo*, filled squares; or *trans*-only, open circles) or ATP and GroES (wild-type GroEL, filled circles), and MDH assays carried out at various times as described in Figure 4C. (E) Rescue of the GroES–GroEL-deficient *E. coli* strain, LG6, by transformation with various GroEL- and GroES-encoding plasmids, including *transin vivo*. Colony sizes are scored relative to wild-type (rated as 10) (see text). (F) Expression of GroEL proteins in rescued LG6 colonies. Representative individual colonies from the experiment in (E) were dispersed in SDS sample buffer, the soluble material was separated by SDS-PAGE, and the gel was stained. Rescuing constructs: lane 1, ES + dimer; lane 2, *transin vivo*; lane 3, ES + *transin vivo*; lane 4, wild-type ES + EL. The right-hand lane contains standard proteins. GroES (10 kDa) is not observed because it migrates at the dye front of this 6% SDS-gel.

in side view images, with the proximate apical domains remaining in a non-elevated position (Figure 6C, left images). After addition of ADP, the classic bullet-shaped images, with GroEL apical domains elevated in the GroES-bound ring, were seen in side views, as had been seen with the *trans*-only complex (Figure 6C, right images). To establish that substrate could not enter the GroES-apposed GroEL ring (U2), a proteinase K protection assay was performed, using radiolabeled MDH as

substrate. As before with *trans*-only, no MDH was protected upon ADP addition, reflecting that none had been encapsulated underneath the tethered U2 ring (not shown). *Transin vivo* was also able to support MDH refolding in the presence of ATP, with a rate and extent similar to *trans*-only (Figure 6D).

The *transin vivo* plasmid was tested for its ability to rescue the GroEL–GroES-deficient strain LG6, carrying a single chromosomal copy of the *GroE* operon under *lac*

regulation (Horwich *et al.*, 1993). When plated in the absence of isopropyl- β -D-thiogalactopyranoside (IPTG), this strain fails to make colonies. LG6 cells were transformed with equal amounts of plasmid DNA directing expression of GroES, GroEL and derivatives from a leaky *trc* promoter (Figure 6E) and plated at 25°C in the absence of IPTG. Growth was scored as colony size relative to wild-type at 24 and 36 h. Notably, wherever colonies appeared, they were present in the same numbers, but the sizes of the colonies, uniform for any given construct and scored on a scale of 1–10, differed substantially.

As shown in Figure 6E, the parental dimer construct produces colonies the same size as wild-type GroES–GroEL when co-expressed with GroES (ES + dimer). As expected, the dimer construct fails to produce any colonies in the absence of a co-expressed GroES (dimer). Correspondingly, in the presence of co-expressed GroES, *transin vivo* produces colonies about one-third to one-half the size of those of wild-type GroEL or dimer (ES + *transin vivo*). Here, the added presence of non-tethered GroES would allow *cis* folding to occur by encapsulation of substrates in the non-tethered (U1) ring (see Figure 6B). The somewhat reduced colony size relative to wild-type and ES + dimer may relate to the fraction of *transin vivo* molecules that misfold or aggregate, limiting the level of functional GroEL molecules. In contrast to dimer alone, however, *transin vivo* alone, in the absence of co-expressed GroES, produced a lawn of small colonies approximately one-tenth the size of wild-type and one-quarter the size of ES + *transin vivo*. This indicates that the *trans* reaction can function sufficiently *in vivo* to support viability of LG6 cells, i.e. that folding of all of the essential GroEL substrate proteins can proceed at a level sufficient to allow cell growth. Yet, as with its behavior *in vitro*, the *trans*-only reaction appeared to support folding *in vivo* at lower efficiency than *cis*, directly demonstrated by comparing colony size of ES + *transin vivo* with that of *transin vivo* alone.

We were concerned that the rescue by *transin vivo* might result from cleavage of the GroES moiety from the subunit fusion, allowing free GroES to assemble and function *in cis*. Direct analysis of rescued LG6 colonies by SDS–PAGE, however, revealed only full-length molecules, migrating characteristically more slowly than dimer (Figure 6F, lanes 2 and 3). In a further analysis of the *transin vivo* construct, a hemagglutinin (HA) epitope was added to the C-terminus of the GroES moiety, and cell extracts were gel filtered, and immunoblotted with anti-HA to detect cleavage and reassembly of free GroES. No immunoreactive species were found in fractions where free GroES complexes would have migrated (~75 kDa) (see Supplementary data and figure).

Discussion

Selectivity of *cis* and *trans* folding by GroEL–GroES

The foregoing studies with *trans*-only GroEL–GroES complexes both *in vitro* and *in vivo* make clear that the *trans* mechanism can function beyond assisting large substrate proteins, such as aconitase. Here, the *trans* mechanism mediated refolding *in vitro* of two stringently GroEL–GroES-dependent substrate proteins, Rubisco and

MDH, whose folding had been inferred to occur only by the *cis* mechanism. In addition, a *trans*-only complex was sufficient *in vivo* to support folding of all of the essential *E.coli* proteins requiring GroEL–GroES, allowing growth of an otherwise GroEL–GroES-deficient strain. The *trans* reaction was slower than *cis in vitro*, and cell growth *in vivo* was similarly slower than with wild-type, probably reflecting a less efficient folding process for substrates that normally fold via a *cis* mechanism. Assuming that the same nucleotide cycle is operative for both *cis* and *trans* reactions, as appears to be the case given identical steady-state rates of ATP turnover (G.W.Farr, unpublished), the chances of a bound Rubisco molecule reaching the native state are at least 4-fold greater for release into the GroES-encapsulated chamber *in cis* than for release into the bulk solution occurring *in trans*. Yet the *trans* reaction is steadily productive, and folding of a given number of Rubisco molecules (up to 50%) can eventually occur via an ~4-fold greater number of cycles than with *cis*. As with *cis* folding, we presume that any individual *trans* cycle represents an all-or-none trial at folding. It seems likely that this attempt at reaching native form occurs in the bulk solution following release from the *trans* ring (see below), and that rebinding of forms that remain non-native may be associated with an action of unfolding that returns them to the original unfolded state(s) (Chan and Dill, 1996; Todd *et al.*, 1996; Zahn *et al.*, 1996).

Insofar as the *trans* reaction supported folding of both a larger substrate that could not be folded in *cis*, as well as smaller GroEL–GroES-dependent substrates that are able to be folded *in cis*, it appears to supply a universal GroEL–GroES folding function. It is tempting to speculate that, evolutionarily, the *trans* mechanism may have antedated the *cis* mechanism, with early GroES analogs functioning as release factors that could act generally *in trans* to eject substrates of all sizes. Regardless of the evolutionary trajectory, in the modern situation it seems clear that smaller proteins, able to be encapsulated *in cis*, would be largely committed to using the *cis* pathway, given the rapid ATP-driven arrival of GroES onto open polypeptide-bound GroEL rings (Rye *et al.*, 1999) and the significantly greater efficiency of *cis* folding. However, for larger substrate proteins and even for smaller mass proteins that have an asymmetric geometry in the non-native state that cannot be encapsulated in the *cis* cavity, the *trans* mechanism is the only GroEL–GroES-driven pathway that can be taken. How many such substrates rely on this pathway is unknown at present. We note the recent discovery from studies of Hsp60- and Hsp10-deficient yeast mitochondria of an additional substrate, the 84 kDa mitochondrial intermediate protease, or octapeptidase (H.Otto and S.Rospert, personal communication). Proteomic techniques in mitochondria and *E.coli* should be able to resolve further the collective of obligate *trans* substrates.

Comparison of mechanisms of folding with and without encapsulation—importance of rounds of rebinding

The observations here that stringent GroEL–GroES-dependent substrate proteins could be folded productively in the absence of *cis* encapsulation both *in vitro* and *in vivo* comes as a surprise. In an earlier study, for example, when

the substrate ornithine transcarbamylase was tested *in vitro* in a *trans* reaction, no productive folding was observed (Weissman *et al.*, 1995). That experiment employed ADP instead of ATP, however, and was confined to a single cycle. Here, in contrast, the *trans* reactions were allowed to proceed through multiple cycles in ATP, the physiological nucleotide. Nevertheless, it is surprising that the *trans* reaction can be productive at all, because it lacks such favorable features of the *cis* folding chamber as solitary confinement and hydrophilic cavity walls, as well as the 10 s of time for folding within this cavity during each round. Instead of these, polypeptide is ejected from an open GroEL ring directly into the bulk solution at each round of the *trans* mechanism. This environment is unproductive for refolding of Rubisco or MDH when they are diluted from denaturant *in vitro* (e.g. Goloubinoff *et al.*, 1989; Ranson *et al.*, 1995). *In vivo*, likewise, the bulk cytoplasm or mitochondrial matrix is an unfavorable environment for spontaneous folding of chaperonin-dependent substrates, as studies with Hsp60-deficient mitochondria have made clear (Cheng *et al.*, 1989; Dubaquié *et al.*, 1998). Yet the *trans* reaction was able to support refolding *in vitro* and cell viability *in vivo*, albeit with reduced efficiency.

Productivity of *trans* folding probably relies on two critical features. One is the ability of the chaperonin to bind recursively the non-native forms that have not achieved the native state after ejection into the bulk solvent, amounting to >99% of the *trans*-released species of MDH or Rubisco at any given cycle. Because the ejected polypeptide faces a kinetic competition between productive folding and rebinding to an open GroEL ring, it is surprising that rebinding of substrates from *trans* does not always prevail over productive folding, preventing any molecules from reaching the native state. In this regard, we asked whether increasing the concentration of *trans*-only by 25-fold (i.e. to 50 μ M versus 2 μ M used in Figure 4C) would further slow the kinetics of refolding of MDH (1 μ M), but no effect was observed. This leaves us to conclude that commitment to reaching the native form has already occurred for any *trans*-folding molecule at the point of release. This is likely to be a function of an ensemble of non-native states of a substrate protein being bound to a population of GroEL molecules, with only a small fraction (<1%) capable of reaching native form if released. In contrast, in the case of *cis* folding, commitment to reaching native form can occur even after the point of release into the *cis* cavity, during the ~10 s lifetime of this state. This is demonstrated most clearly with long-lived *cis* complexes (with single ring or hydrolysis-defective GroEL), in which an entire population of non-native molecules can reach native form after a single round of release into the cavity if given enough time (Weissman *et al.*, 1996; Rye *et al.*, 1997).

The other feature of *trans* folding that is likely to be crucial to its productivity is the step of release, effective only in the presence of ATP/GroES and not ATP alone. Earlier studies indicated that only a small fraction of aconitase was released *in trans* from GroEL by ATP alone (Chaudhuri *et al.*, 2001). Similarly, smaller GroEL–GroES-dependent substrates such as rhodanese are released inefficiently by ATP alone (Weissman *et al.*, 1994). Kinetic studies of ATP hydrolysis have indicated

that ATP/GroES binding indeed promotes an allosteric transition of the distal ring to a state that exhibits low affinity for polypeptide, suggested to serve potentially the purpose of releasing substrate proteins (Inbar and Horovitz, 1997; Makio *et al.*, 1999). At a structural level, how is this accomplished? Does GroES binding produce a qualitatively different allosteric conformational adjustment of the opposite polypeptide-bearing ring than does ATP alone? For example, is there a greater excursion of *trans* apical domain movement? Or is ATP/GroES serving to coordinate concerted conformational change of all seven subunits of the opposite ring, allowing a simultaneous release of polypeptide from all of its sites of apical binding? Ongoing cryoEM studies of ATP/GroES-bound GroEL complexes should be able to resolve the conformation of the *trans* ring and allow comparison with the ATP-bound state (Ranson *et al.*, 2001), addressing these questions.

Materials and methods

Constructs and proteins

Trans-only GroEL–GroES. GroEL315C and GroES₁₀₄ (or GroES₁₁₀) were exchanged into 50 mM HEPES, 5 mM TCEP [tris(2-carboxyethyl)phosphine] pH 7.4, by dilution and centrifugal concentration (Amicon Ultra) and combined in 50 mM HEPES, 10 mM KCl, 10 mM MgCl₂, 5 mM TCEP pH 7.4, to give 20 μ M GroEL315C and 40 μ M GroES₁₀₄ (or GroES₁₁₀). ADP was added to 10 mM, and the mixture was incubated for 30 min at room temperature to form a binary complex. Cross-linker BM(PEO)₃ (Pierce) was added to 16 mM (~5-fold excess over total -SH), and the incubation was continued overnight. *Trans*-only complex was separated from excess GroES₁₀₄ and reagents by anion exchange chromatography. The presence of cross-linked subunits was confirmed by SDS–PAGE and mass spectrometry. A similar protocol was used to cross-link SR315C and GroES₁₀₄ to produce SR-X-ES.

Variant GroES, transin vivo GroEL–GroES and other proteins. For these methods, see Supplementary data.

Assays

Substrate proteins were unfolded, refolded, and assayed as previously reported (aconitase, Chaudhuri *et al.*, 2001; MDH and Rubisco, Rye *et al.*, 1997). For refolding of aconitase with ADP–AlF_x, an ordered addition was used: 5 mM ADP added first to chaperonin–aconitase binary complex for 30 min, followed by 30 mM KF for 5 min, then 3 mM KAl(SO₄)₂. Radiolabeling of cross-linker, ‘hit-and-run’ cross-linking and proteinase K treatment of GroEL complexes were carried out essentially as before (Weissman *et al.*, 1995).

EM methods

CryoEM images were collected using a 200 kV field emission gun microscope and digitized as previously described (Ranson *et al.*, 2001) and negative stain images were collected on a 120 kV microscope with low electron dose. Pre-processing was with MRC software (Crowther *et al.*, 1996), and alignment and image classification were with Imagic (van Heel *et al.*, 1996). Class averages from cryoEM showing obvious extra density contained 648 out of 1485 images in the data set for *trans*-only and 280 out of 1300 images for *transin vivo*. The extra density is not easily detectable on the raw images, and this feature is often not aligned and sorted consistently. All negative stain side views of the complexes with ADP showed the typical bullet-shaped structure (110 images of *trans*-only and 70 of *transin vivo*) (see Supplementary data for additional description).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank Neil Ranson for help with EM analyses. This work was supported by the Wellcome Trust, NIH and HHMI.

References

- Brinker, A., Pfeifer, G., Kerner, M.J., Naylor, D.J., Hartl, F.U. and Hayer-Hartl, M. (2001) Dual function of protein confinement in chaperonin-assisted protein folding. *Cell*, **107**, 223–233.
- Chaudhuri, T.K., Farr, G.W., Fenton, W.A., Rospert, S. and Horwich, A.L. (2001) GroEL/GroES-mediated folding of a protein too large to be encapsulated. *Cell*, **107**, 235–246.
- Chan, H.S. and Dill, K.A. (1996) A simple model of chaperonin-mediated protein folding. *Proteins*, **24**, 345–351.
- Cheng, M.Y., Hartl, F.-U., Martin, J., Pollock, R.A., Kalousek, F., Neupert, W., Hallberg, E.M., Hallberg, R.L. and Horwich, A.L. (1989) Mitochondrial heat-shock protein Hsp60 is essential for assembly of proteins imported into yeast mitochondria. *Nature*, **337**, 620–625.
- Crowther, R.A., Henderson, R. and Smith, J. (1996) MRC image processing programs. *J. Struct. Biol.*, **116**, 9–16.
- Dubaquíé, Y., Looser, R., Fünfschilling, U., Jenö, P. and Rospert, S. (1998) Identification of *in vivo* substrates of the yeast mitochondrial chaperonins reveals overlapping but non-identical requirement for hsp60 and hsp10. *EMBO J.*, **17**, 5868–5876.
- Farr, G.W., Furtak, K., Rowland, M.B., Ranson, N.A., Saibil, H.R., Kirchhausen, T. and Horwich, A.L. (2000) Multivalent binding of nonnative substrate proteins by the chaperonin GroEL. *Cell*, **100**, 561–573.
- Goloubinoff, P., Christeller, J.T., Gatenby, A.A. and Lorimer, G.H. (1989) Reconstitution of active dimeric ribulose biphosphate carboxylase from an unfolded state depends on two chaperonin proteins and MgATP. *Nature*, **342**, 884–889.
- Grantcharova, V., Alm, E.J., Baker, D. and Horwich, A.L. (2001) Mechanisms of protein folding. *Curr. Opin. Struct. Biol.*, **11**, 70–82.
- Hartl, F.U. and Hayer-Hartl, M. (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science*, **295**, 1852–1858.
- Horwich, A.L., Low, K.B., Fenton, W.A., Hirshfield, I.N. and Furtak, K. (1993) Folding *in vivo* of bacterial cytoplasmic proteins: role of GroEL. *Cell*, **74**, 909–917.
- Huang, Y.-S. and Chuang, D.T. (1999) Mechanisms for GroEL/GroES-mediated folding of a large 86-kDa fusion polypeptide *in vitro*. *J. Biol. Chem.*, **274**, 10405–10412.
- Inbar, E. and Horovitz, A. (1997) GroES promotes the T to R transition of the GroEL ring distal to GroES in the GroEL–GroES complex. *Biochemistry*, **36**, 12276–12281.
- Langer, T., Pfeifer, G., Martin, J., Baumeister, W. and Hartl, F.-U. (1992) Chaperonin-mediated protein folding: GroES binds to one end of the GroEL cylinder, which accommodates the protein substrate within its central cavity. *EMBO J.*, **11**, 4757–4765.
- Makio, T., Arai, M. and Kuwajima, K. (1999) Chaperonin-affected refolding of α -lactalbumin: effects of nucleotides and the co-chaperonin GroES. *J. Mol. Biol.*, **293**, 125–137.
- Mayhew, M., da Silva, A.C., Martin, J., Erdjument-Bromage, H., Tempst, P. and Hartl, F.U. (1996) Protein folding in the central cavity of the GroEL–GroES chaperonin complex. *Nature*, **379**, 420–426.
- Ranson, N.A., Dunster, N.J., Burston, S.G. and Clarke, A.R. (1995) Chaperonins can catalyze the reversal of early aggregation steps when a protein misfolds. *J. Mol. Biol.*, **250**, 581–586.
- Ranson, N.A., Burston, S.G. and Clarke, A.R. (1997) Binding, encapsulation and ejection: substrate dynamics during a chaperonin-assisted folding reaction. *J. Mol. Biol.*, **266**, 656–664.
- Ranson, N.A., Farr, G.W., Roseman, A.M., Gowen, B., Fenton, W.A., Horwich, A.L. and Saibil, H.R. (2001) ATP-bound states of GroEL captured by cryo-electron microscopy. *Cell*, **107**, 869–879.
- Rye, H.S., Burston, S.G., Fenton, W.A., Beechem, J.M., Xu, Z., Sigler, P.B. and Horwich, A.L. (1997) Distinct actions of *cis* and *trans* ATP within the double ring of the chaperonin GroEL. *Nature*, **388**, 792–798.
- Rye, H.S., Roseman, A.M., Chen, S., Furtak, K., Fenton, W.A., Saibil, H.R. and Horwich, A.L. (1999) GroEL–GroES cycling: ATP and non-native polypeptide direct alternation of folding-active rings. *Cell*, **97**, 325–328.
- Smith, K.E. and Fisher, M.T. (1995) Interactions between the GroE chaperonins and rhodanese. Multiple intermediates and release and rebinding. *J. Biol. Chem.*, **270**, 21517–21523.
- Thirumalai, D. and Lorimer, G.H. (2001) Chaperonin-mediated protein folding. *Annu. Rev. Biophys. Biomol. Struct.*, **30**, 245–269.
- Todd, M.J., Viitanen, P.V. and Lorimer, G.H. (1994) Dynamics of the chaperonin ATPase cycle: implications for facilitated protein folding. *Science*, **265**, 659–666.
- Todd, M.J., Lorimer, G.H. and Thirumalai, D. (1996) Chaperonin-facilitated protein folding: optimization of rate and yield by an iterative annealing mechanism. *Proc. Natl Acad. Sci. USA*, **93**, 4030–4035.
- van Heel, M., Harauz, G., Orlova, E., Schmidt, R. and Schatz, M. (1996) A new generation of the IMAGIC image processing system. *J. Struct. Biol.*, **116**, 17–24.
- Weissman, J.S., Kashi, Y., Fenton, W.A. and Horwich, A.L. (1994) GroEL-mediated protein folding proceeds by multiple rounds of binding and release of nonnative forms. *Cell*, **78**, 693–702.
- 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) Mechanism of GroEL action: productive release of polypeptide from a sequestered position under GroES. *Cell*, **83**, 577–587.
- Weissman, J.S., Rye, H.S., Fenton, W.A., Beechem, J.M. and Horwich, A.L. (1996) Characterization of the active intermediate of a GroEL–GroES-mediated protein folding reaction. *Cell*, **84**, 481–490.
- Xu, Z., Horwich, A.L. and Sigler, P.B. (1997) The crystal structure of the asymmetric GroEL–GroES-(ADP)₇ chaperonin complex. *Nature*, **388**, 741–750.
- Zahn, R., Perrett, S. and Fersht, A.R. (1996) Conformational states bound by the molecular chaperones GroEL and SecB: a hidden unfolding (annealing) activity. *J. Mol. Biol.*, **261**, 43–61.

Received March 11, 2003; revised May 2, 2003;
accepted May 7, 2003