The EMBO Journal vol.15 no.22 pp.6111–6121, 1996

Mechanism of chaperonin action: GroES binding and release can drive GroEL-mediated protein folding in the absence of ATP hydrolysis

Manajit K.Hayer-Hartl, Frank Weber and F.Ulrich Hartl[1]

Howard Hughes Medical Institute and Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA

[1]Corresponding author

As a basic principle, assisted protein folding by GroEL has been proposed to involve the disruption of misfolded protein structures through ATP hydrolysis and interaction with the cofactor GroES. Here, we describe chaperonin subreactions that prompt a re-examination of this view. We find that GroEL-bound substrate polypeptide can induce GroES cycling on and off GroEL in the presence of ADP. This mechanism promotes efficient folding of the model protein rhodanese, although at a slower rate than in the presence of ATP. Folding occurs when GroES displaces the bound protein into the sequestered volume of the GroEL cavity. Resulting native protein leaves GroEL upon GroES release. A single-ring variant of GroEL is also fully functional in supporting this reaction cycle. We conclude that neither the energy of ATP hydrolysis nor the allosteric coupling of the two GroEL rings is directly required for GroEL/GroES-mediated protein folding. The minimal mechanism of the reaction is the binding and release of GroES to a polypeptide-containing ring of GroEL, thereby closing and opening the GroEL folding cage. The role of ATP hydrolysis is mainly to induce conformational changes in GroEL that result in GroES cycling at a physiologically relevant rate.

Keywords: chaperonins/GroEL/GroES/molecular chaperones/protein folding

Introduction

The chaperonins GroEL and GroES mediate the efficient folding of many newly synthesized polypeptides in the bacterial cytosol in an ATP-dependent reaction (reviewed in Ellis, 1994b; Hendrick and Hartl, 1995; Hartl, 1996). GroEL is a cylindrical protein complex of 14 subunits of 57 kDa which are arranged in two heptameric rings stacked back-to-back. Substrate protein binds in the central cavity of the cylinder in the conformation of an unstable, yet compact folding intermediate that exposes hydrophobic surfaces. Recent crystallographic analysis has revealed a three-domain structure of the GroEL subunits (Braig *et al.*, 1994); the equatorial domain contains the ATP binding site and provides most of the inter-subunit contacts within and between heptameric rings. The flexible apical domain forms the opening of the cylinder and exposes a number of hydrophobic residues towards the central cavity which provide a binding surface for unfolded polypeptide. The apical domain is connected to the equatorial domain via a hinge-like intermediate domain. GroES, a single ring of seven subunits, each of ~10 kDa (Hunt *et al.*, 1996; Mande *et al.*, 1996), is essential to the mechanism of GroEL-assisted protein folding (Martin *et al.*, 1991; Schmidt *et al.*, 1994a). Under most conditions in the presence of ADP and ATP, GroES forms an asymmetrical complex with GroEL, capping one end of the GroEL cylinder (Langer *et al.*, 1992; Saibil *et al.*, 1993; Chen *et al.*, 1994; Engel *et al.*, 1995). In addition, symmetrical complexes with two GroES rings bound to either side of GroEL can be observed at elevated concentrations of Mg^{2+}-ATP (Azem *et al.*, 1994; Llorca *et al.*, 1994; Schmidt *et al.*, 1994b). GroES binding causes a dramatic upwards and outwards movement of the apical GroEL domains, thereby increasing the size of the central cavity and forming a dome-shaped chamber ~65 Å high and 80 Å wide (Chen *et al.*, 1994). Regions of the apical domains involved in polypeptide binding also participate in the interaction with GroES (Fenton *et al.*, 1994).

Based on recent advances, two distinct mechanisms of GroES action have been proposed. (i) The role of GroES is to increase the cooperativity of the GroEL ATPase in such a manner that ATP hydrolysis becomes 'quantized'; i.e. all seven ATPs occupying a single GroEL ring are hydrolyzed in a coordinated manner. The energy of quantized ATP hydrolysis is then available for the disruption of misfolded protein structures (Todd *et al.*, 1994, 1996). Folding itself can occur in free solution upon ejection of unfolded polypeptide from GroEL (Todd *et al.*, 1994; Weissman *et al.*, 1994). (ii) By binding to the GroEL ring that holds the unfolded protein (Martin *et al.*, 1993), GroES causes the displacement of the bound polypeptide from its hydrophobic attachment sites into the enlarged ring cavity for folding in a sequestered environment (Hartl, 1994; Mayhew *et al.*, 1996; Weissman *et al.*, 1996). In this model, ATP hydrolysis by GroEL regulates the timed dissociation of the GroEL–GroES complex so that folded protein can leave the GroEL cavity. The rearrangement of misfolded or kinetically trapped folding intermediates would be mediated by the re-binding of polypeptide to GroEL, when GroES is released. Indeed, folding in the GroEL cavity has been demonstrated for several proteins (Gray and Fersht, 1993; Corrales and Fersht, 1995; Mayhew *et al.*, 1996; Weissman *et al.*, 1996).

In order to evaluate the mechanistic significance of ATP hydrolysis by GroEL, we analyzed the consequences of GroES binding and release to a GroEL–polypeptide complex in the presence of non-hydrolyzable nucleotides. We find that substrate polypeptide alone can induce the cycling of GroES between GroEL-bound and free states in the presence of ADP, resulting in efficient folding in

the GroEL cavity underneath GroES. This basic reaction can be carried out by a single ring of GroEL. Our results support the view that the energy of ATP hydrolysis is not used directly to drive protein folding or unfolding, but rather to induce conformational changes in GroEL that increase the rate of GroES cycling under certain conditions.

Results

GroES encloses rhodanese in the GroEL cavity

Recent studies suggested that productive protein folding by GroEL involves the transient enclosure of the substrate protein within the GroEL cavity by GroES (see references above). This has been established by measuring the protease protection of GroEL-bound polypeptide upon binding of GroES to the polypeptide-containing ring of GroEL (the so-called *cis*-topology; see Figure 1A) (Weissman *et al.*, 1995; Mayhew *et al.*, 1996). To demonstrate this with rhodanese as the substrate, a GroEL–rhodanese complex was prepared by diluting denatured rhodanese from 6 M guanidinium hydrochloride into buffer solution containing GroEL, followed by the isolation of the complex by size-exclusion chromatography. Bovine rhodanese (thiosulfate sulfurtransferase; EC 2.8.1.1) was chosen as a model substrate, because under the experimental conditions this protein aggregates upon attempted spontaneous refolding. Renaturation of rhodanese by GroEL is strictly dependent on GroES (Martin *et al.*, 1991; Mendoza *et al.*, 1991) and involves multiple turnovers of the GroEL ATPase (Martin *et al.*, 1991).

Addition of proteinase K to the isolated GroEL–rhodanese complex in the absence of GroES rapidly digested all the GroEL-bound protein under conditions in which GroEL itself remained intact, except for the cleavage of the 16 C-terminal amino acid residues from each GroEL subunit (not shown) (Langer *et al.*, 1992; Martin *et al.*, 1993). These residues form flexible tails protruding into the central cavity of the chaperonin from the equatorial domains (Braig *et al.*, 1994). Their removal does not impair the structure or function of GroEL. In contrast, up to 40% of the GroEL-bound rhodanese was protected against proteolysis when GroES was added to the complex in the presence of ADP (Figure 1A and B). This level of protection is expected, because GroES binds with similar efficiency either to the free or the polypeptide-containing ring of the GroEL–polypeptide complex, resulting in a mixture of *trans*- and *cis*-complexes, respectively (Martin *et al.*, 1993; Weissman *et al.*, 1995, 1996; Mayhew *et al.*, 1996). The same observation was made on binding GroES in the presence of the non-hydrolyzable ATP analog AMP–PNP (Figure 1C).

GroES-dependent folding in the absence of ATP hydrolysis

Most of the rhodanese protein that was enclosed in the GroEL cavity by GroES in the presence of ADP or AMP–PNP reached the native state upon subsequent incubation of the protease-treated chaperonin complex with ATP (Figure 1C). Interestingly, a significant amount of enzymatically active rhodanese was already present prior to the addition of ATP (not shown) and must have folded during the incubation of the GroEL–rhodanese–GroES complex

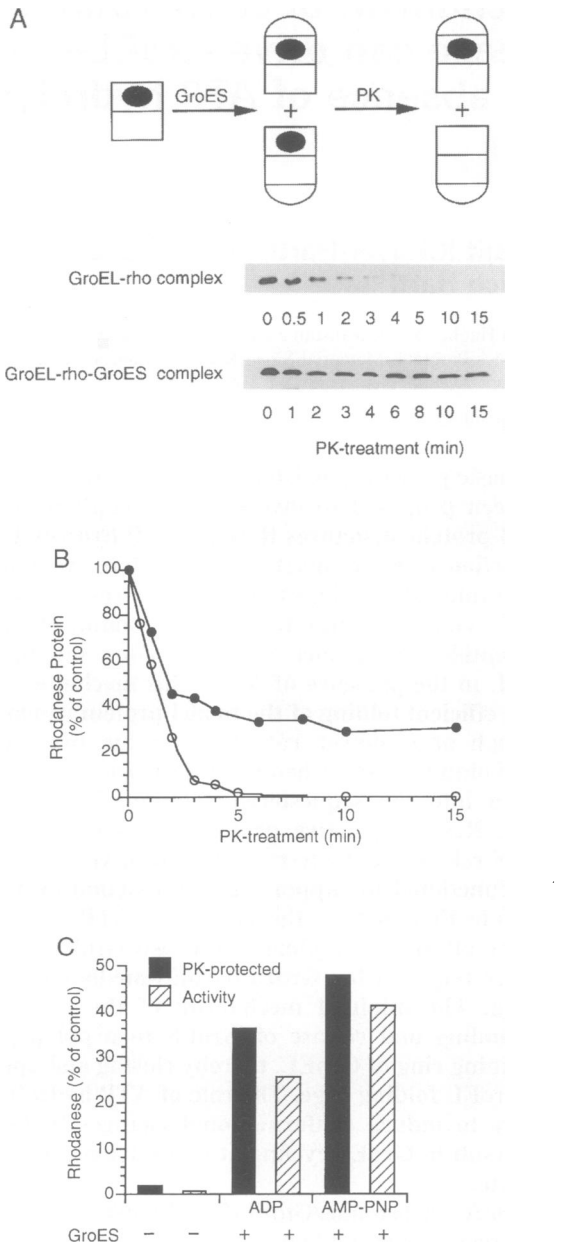

Fig. 1. Protease protection of GroEL-bound rhodanese by GroES. (**A**) Isolated GroEL–rhodanese complex (6.5 μM GroEL 14-mer) was incubated in buffer A/5 mM Mg-acetate containing 0.2 mM ADP with 11 μM GroES and then treated with proteinase K (10 μg/ml) for 0–15 min at 25°C. Proteinase K action was stopped with 1 mM PMSF. GroEL was analyzed by SDS–PAGE on a 12.5% acrylamide gel. Half of each reaction was transferred to nitrocellulose and immunoblotted with rhodanese antibodies (lower panel). PK, proteinase K; rho, rhodanese. The upper panel shows schematically the two possibilities of GroES binding: GroES interacts either in *cis*-topology with the ring of GroEL that contains the bound rhodanese (black sphere) or in *trans*-topology with the opposite ring. Only the interaction in *cis* results in protease protection of rhodanese (Weissman *et al.*, 1994; Mayhew *et al.*, 1996). (**B**) Quantitation of undigested rhodanese in (A) by densitometry. GroEL–rhodanese (○) and GroEL–rhodanese–GroES (●). Amounts of bound rhodanese in the absence of PK are set to 100%. (**C**) GroEL–rhodanese complex in 0.2 mM ADP or 5 mM AMP–PNP as in (A) was treated with PK for 5 min at 25°C. Amounts of undigested rhodanese were quantified by densitometry of immunoblots and rhodanese activities were measured as in Materials and methods after incubation of the complex with 5 mM ATP for 60 min. Amounts of rhodanese protein and of rhodanese activity in the non-protease treated reaction are set to 100%.

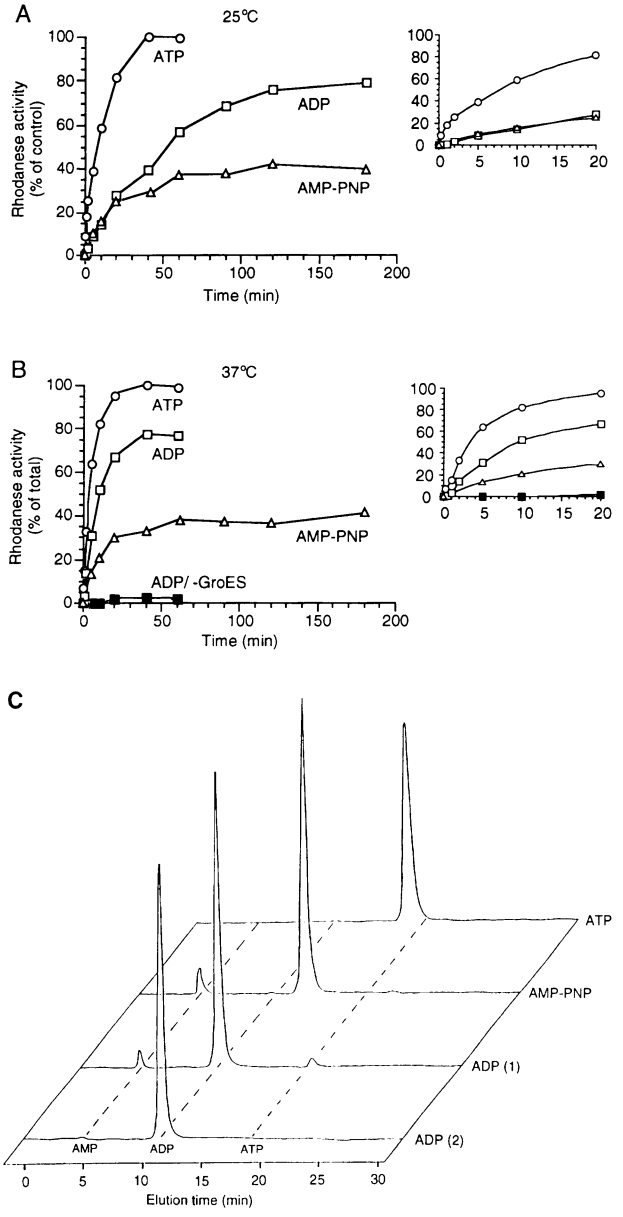

Fig. 2. Nucleotide and GroES-dependent reactivation of GroEL-bound rhodanese. (**A** and **B**) Isolated GroEL–rhodanese complex (3–4 μM) was incubated with 8 μM GroES at (A) 25°C or (B) 37°C with 5 mM ATP (○); 0.2 mM ADP (□) or 5 mM AMP–PNP (Δ). Folding was initiated by addition of the respective nucleotide. At the times indicated, rhodanese activities were determined for 15 min (A) or 3 min (B). A control reaction in (B) was incubated with 0.2 mM ADP (■) in the absence of GroES. Inserts show time-courses of reactions for the first 20 min. Activities are expressed as a percentage of the maximum activity reached in the presence of ATP. (**C**) Analysis of adenine nucleotides by anion exchange chromatography. Absorbances at 280 nm versus elution time are shown for ATP, AMP–PNP, ADP (1) and ADP (2).

with ADP or AMP–PNP. Furthermore, when a GroEL–rhodanese complex, not treated with proteinase K, was incubated with GroES and ADP, rhodanese renatured with ~80% efficiency compared with an ATP-driven control reaction (Figure 2A). Folding was completely dependent on both GroES and a physiological concentration of ADP (0.2 mM), and occurred with a half-time of ~40 min at 25°C, i.e. at an apparent rate ~8-fold slower than the ATP-mediated reaction. The rate of ADP-dependent folding increased at 37°C to a rate only ~2-fold slower than folding in the presence of ATP (Figure 2B). These findings were unexpected, because the chaperonin-mediated folding of rhodanese is thought to be dependent on both GroES and ATP hydrolysis. We therefore analyzed the purity of the various nucleotides by FPLC ion exchange chromatography (Figure 2C). The ADP solution (trace 2), used in the refolding experiments, was essentially pure, while the AMP–PNP contained a minor contaminant that fractionated like AMP.

Rhodanese folding was also observed when GroES was bound to a GroEL–rhodanese complex in the presence of a saturating concentration of AMP–PNP (5 mM) (Figure 2). In this case, however, the maximal yield of active rhodanese did not exceed 40%, suggesting that only those molecules enclosed in the GroEL cavity upon GroES binding folded. At 25°C, the rate of AMP–PNP-mediated folding was the same as that in the presence of ADP (Figure 2A), but did not increase significantly at 37°C (Figure 2B and Figure 2, inserts). The high yield of ADP-mediated renaturation was inconsistent with folding being restricted to the ~40% of rhodanese molecules that were enclosed within the GroEL cavity upon initial binding of GroES. Since folding of rhodanese occurs only from the GroES *cis*-complex (Weissman *et al.*, 1995), GroES must have cycled between GroEL-bound and free states. However, this seemed to contradict the well-documented stability and the strictly asymmetric nature of the GroEL–GroES complex in the presence of ADP (Jackson *et al.*, 1993; Martin *et al.*, 1993; Todd *et al.*, 1993; Burston *et al.*, 1995; Hayer-Hartl *et al.*, 1995). To resolve this problem, we first tested whether folded rhodanese was released from GroEL upon incubation with ADP. A complex of GroEL with unfolded ^{3}H-labelled rhodanese (Hlodan *et al.*, 1995) was formed and separated from non-bound rhodanese. This complex was then incubated for 10 min at 37°C either with ATP and GroES or ADP and GroES, and then immediately analyzed by size-exclusion chromatography in the presence of ADP (Figure 3A and B). In both cases essentially all the enzymatically active ^{3}H-labelled rhodanese fractionated at the position of the free monomeric protein, indicating that GroES must have dissociated from GroEL, even in the presence of ADP, to release rhodanese from the *cis*-ring of GroEL. Similar observations were made at 25°C, but under these conditions rhodanese was released more slowly in the ADP-dependent reaction (not shown).

A different result was obtained upon incubation of the GroEL–[^{3}H]rhodanese complex with GroES and AMP–PNP (Figure 3C). Upon analysis by size-exclusion chromatography in the presence of AMP–PNP, the majority of rhodanese recovered from the column was associated with GroEL (~40% of total). The remaining 60% was released from GroEL but only ~15% fractionated at a position similar to that of the free monomeric protein. This protein was enzymatically inactive (not shown) and apparently represented a kinetically stable folding intermediate or small aggregates of rhodanese. In contrast, more than two-thirds of the GroEL-associated rhodanese was enzymatically active and was apparently enclosed in the GroEL cavity by GroES (see below).

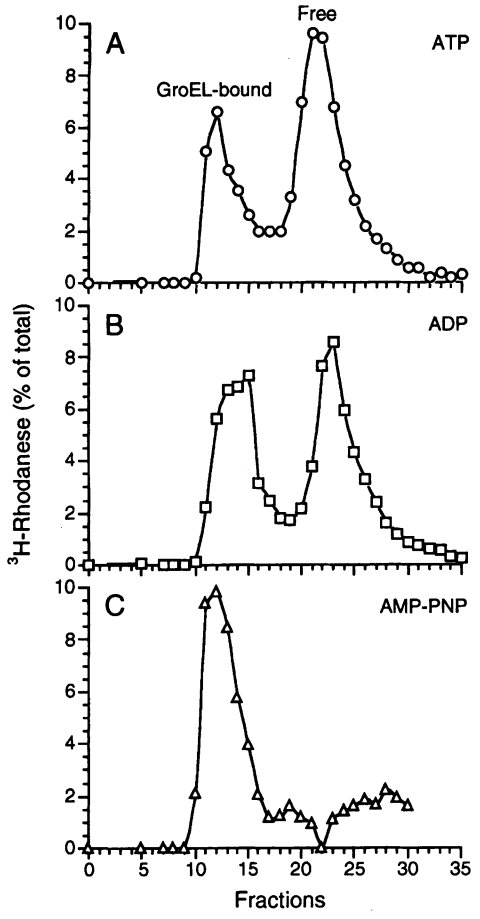

Fig. 3. Release of [^{3}H]rhodanese from GroEL in the presence of (**A**) ATP, (**B**) ADP and (**C**) AMP–PNP. GroES (5 μM) was added to an isolated [^{3}H]rhodanese–GroEL complex (2.5 μM) and refolding was initiated by the addition of either 5 mM ATP, 0.2 mM ADP or 5 mM AMP–PNP. After 10 min at 37°C, the reactions were further incubated in rhodanese enzyme assay (minus CDTA) for 3 min at 37°C and then applied onto a Sepharcryl S300-HR gel filtration column equilibrated in buffer A/5 mM Mg-acetate/0.2 mM ADP (A and B) or 2.5 mM AMP–PNP (C) at 25°C. Fractions of 0.5 ml were collected and analyzed by scintillation counting ([^{3}H]rhodanese) and SDS–PAGE (GroEL, GroES). The peak of GroEL eluted after ~30 min in fraction 12. Amounts of [^{3}H]rhodanese are given in percentage of the total applied. Recoveries of [^{3}H]rhodanese were 81% (A), 83% (B) and 58% (C). All [^{3}H]rhodanese was recovered in the GroEL-bound state (85% of total applied to the column) when the isolated GroEL–[^{3}H]rhodanese complex used as the starting material was separated in the absence nucleotides (not shown).

Substrate-dependent cycling of GroES

GroES is known to cycle between GroEL-bound and free states dependent on ATP hydrolysis by GroEL (Martin *et al.*, 1993; Todd *et al.*, 1994; Burston *et al.*, 1995; Hayer-Hartl *et al.*, 1995). This reaction occurs in the absence of substrate protein. The results described above suggested that a similar cycling is possible without ATP hydrolysis, in the presence of ADP and rhodanese. To establish this directly, we analyzed the exchangeability of ^{3}H-labelled GroES-bound to GroEL, by a 5-fold molar excess of unlabelled GroES (Figure 4). GroES exchange was stopped during a time-course by rapid crosslinking of the GroEL–GroES complex with glutaraldehyde and the amount of GroEL-bound [^{3}H]GroES was analyzed by native polyacrylamide electrophoresis (Figure 4A). As demonstrated previously (Hayer-Hartl *et al.*, 1995), in the

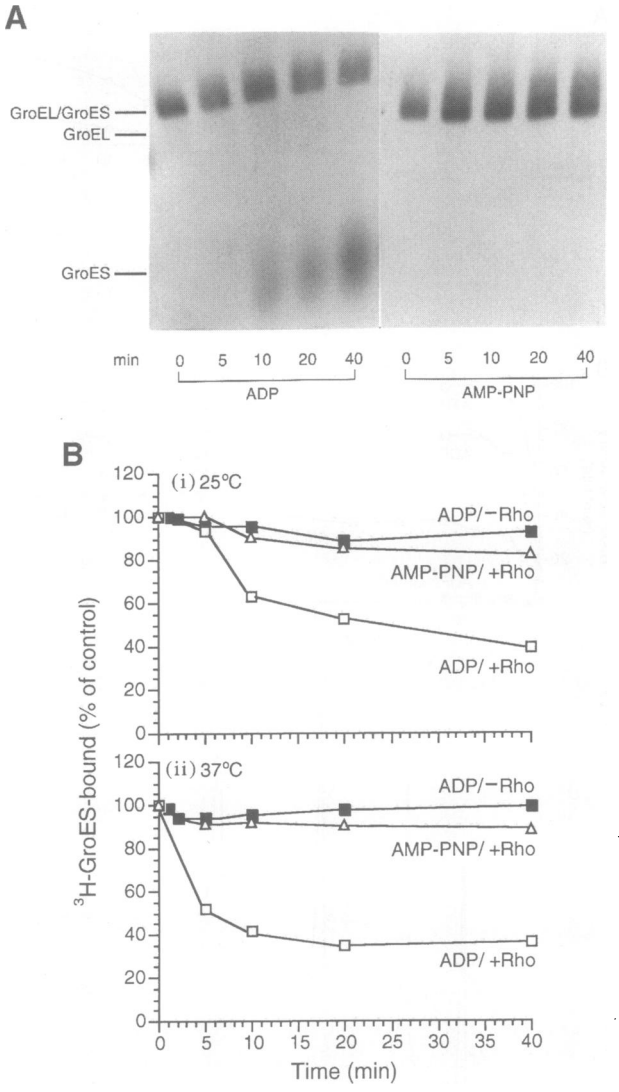

Fig. 4. Exchange of GroEL-bound [^{3}H]GroES by unlabelled GroES. (**A**) GroEL–rhodanese complex (1 μM) was incubated for the times indicated in buffer A/5 mM Mg-acetate with 1 μM [^{3}H]GroES in the presence of 0.2 mM ADP or 5 mM AMP–PNP at 25°C. A 5-fold excess of unlabelled GroES over [^{3}H]GroES was added and, at the times indicated, crosslinked as in Materials and methods. An autoradiograph is shown, indicating the positions of GroEL, GroEL–GroES complex and of free GroES. (**B**) GroEL (–Rho) or GroEL–rhodanese complex (+Rho) was incubated at 1 μM in buffer A/5 mM Mg-acetate with 1 μM [^{3}H]GroES in the presence of 0.2 mM ADP or 5 mM AMP–PNP for 5 min at (i) 25°C or (ii) 37°C. A 5-fold excess of unlabelled GroES over [^{3}H]GroES was added and, at the times indicated, exchange of [^{3}H]GroES by unlabelled GroES analyzed by crosslinking as in (A). Reactions were analyzed by native PAGE and autoradiography. Amounts of GroEL-bound [^{3}H]GroES were quantified by densitometry. [^{3}H]GroES bound to GroEL in the absence of unlabelled GroES was set to 100%.

absence of bound substrate protein, [^{3}H]GroES remained stably bound to GroEL in the presence of ADP at both 25 and 37°C (Figure 4B). In contrast, [^{3}H]GroES that was bound to a preformed GroEL–rhodanese complex in the presence of ADP was exchanged by unlabelled GroES. Exchange, however, did not reach equilibrium, corresponding to ~17% GroEL-bound [^{3}H]GroES, probably because the GroEL in the reaction was not completely saturated with rhodanese (Figure 4A and B). Half of the exchangeable [^{3}H]GroES dissociated from GroEL in ~8–10 min,

indicating that GroES cycling was slow. As a comparison, in the presence of ATP, GroES dissociates from GroEL with a half-time of 15–30 s at 25°C (Hayer-Hartl *et al.*, 1995), the approximate duration of a single reaction cycle. The rhodanese-dependent exchange of [^{3}H]GroES observed in the presence of ADP was ~3-fold faster at 37 than at 25°C (Figure 4B), roughly correlating with the increase in the rate of rhodanese folding at the higher temperature (see Figure 2).

Rhodanese-dependent cycling of GroES was not observed in the presence of AMP–PNP, at 25 or at 37°C (Figure 4A and B). As a result, GroES would have no access to the rhodanese molecules bound to the *trans*-ring of GroEL, explaining the low yield of renaturation compared with folding reactions containing ADP or ATP [symmetrical complexes with GroES bound to either ring of GroEL do not form in the presence of bound rhodanese and AMP–PNP (Engel *et al.*, 1995)]. Furthermore, since the apparent rate of protein folding by GroEL correlated with the rate of GroES release and rebinding, ATP hydrolysis by GroEL mainly appears to accelerate the cycling of GroES, at least with rhodanese as the substrate.

Release of folded and non-folded rhodanese from GroEL

The instability of the GroEL–rhodanese–GroES complex formed in the presence of ADP made it difficult to decide whether rhodanese reached its native state within the GroEL cavity, as occurs in the presence of AMP–PNP. We therefore asked whether rhodanese is released from GroEL in a folded conformation that can no longer interact with chaperonin, the so-called 'committed' state. Two variants of GroEL, a mutant form and an internally glutaraldehyde-crosslinked GroEL, were used in these experiments as 'traps'. Both of these GroEL-traps bind non-native polypeptide with high affinity but are unable to release it, even in the presence of nucleotide and GroES (Weissman *et al.*, 1994; Mayhew *et al.*, 1996). When either GroEL-trap was added to a preformed GroEL–rhodanese complex at a 5-fold molar excess over GroEL, the ATP-dependent refolding of rhodanese was inhibited by ~80% (Figure 5A; only results with mutant GroEL are shown). [Higher concentrations of GroEL-trap do not increase the inhibitory effect on folding (Weissman *et al.*, 1994; Mayhew *et al.*, 1996).] The release of non-native protein set in rapidly in the presence of ATP, apparently as a consequence of the fast rate of GroES cycling under these conditions. Similar observations have previously led to the proposal that GroEL-mediated folding involves the ejection of unfolded protein into the bulk solution where it may fold spontaneously (Todd *et al.*, 1994; Weissman *et al.*, 1994). However, subsequent experiments demonstrated that the release of non-native rhodanese does not yield correctly folded protein and may arise from a leakiness of the GroEL system; each reaction cycle is accompanied by the loss of ~25% of the bound rhodanese in a non-native state, in addition to the release of ~5% protein that folded in the GroEL cavity (Mayhew *et al.*, 1996).

The possibility of uncoupling folding from ATP hydrolysis allowed us to further analyze the significance of the release of non-native polypeptide by GroEL. In contrast to the observations made with folding reactions containing

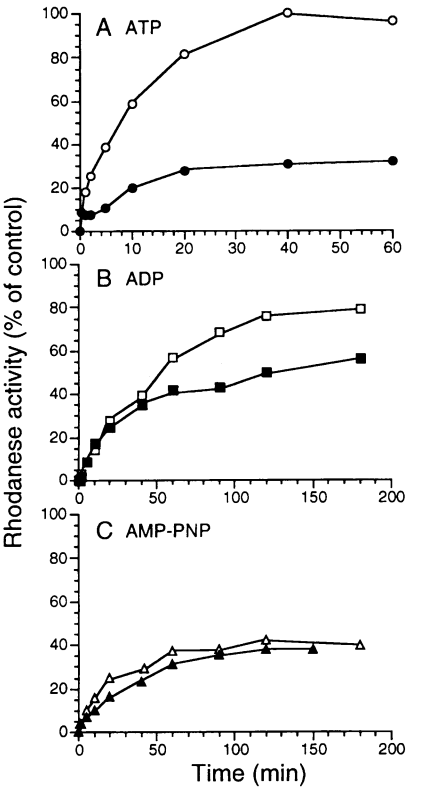

Fig. 5. Effects of GroEL-trap on GroEL-mediated rhodanese folding in the presence of ATP, ADP and AMP–PNP. GroEL–rhodanese complex (1 μM) was incubated in buffer A/5 mM Mg-acetate/50 mM sodium thiosulfate at 25°C in the absence (empty symbols) or presence (filled symbols) of a 5-fold molar excess of GroEL-trap over GroEL and GroES at a 2-fold molar excess over total GroEL. The GroEL-mutant GroEL337/349 (M-GroEL) (Weissman *et al.*, 1994) was used as GroEL-trap in the experiment shown. Identical results were obtained with GA–GroEL (see Materials and methods). Rhodanese refolding was initiated by addition of (**A**) 5 mM ATP (○, ●); (**B**) 0.2 mM ADP (□, ■); or (**C**) 5 mM AMP–PNP (△, ▲). At the times indicated, rhodanese enzyme assays were performed for 10 min at 37°C. Activities measured in 5 mM ATP in the absence of GroEL-trap were set to 100%.

ATP, increasing concentrations of GroEL-trap had much less effect on the rate or yield of rhodanese renaturation observed in the presence of ADP (Figure 5B). A loss of non-native protein in the ADP-containing reaction became detectable only after ~50 min of incubation. Thus, most rhodanese either completed folding in the GroEL cavity or was released in a largely folded, committed state. Consistent with the lack of GroES cycling upon incubation with AMP–PNP, GroEL-trap was without effect on the folding of rhodanese under these conditions (Figure 5C). We conclude that the GroES-dependent folding of rhodanese in the presence of ADP or AMP–PNP proceeds in the GroEL cavity, apparently by the same basic mechanism as folding in the presence of ATP (see Figure 8) (Mayhew *et al.*, 1996). The release of non-native protein from GroEL appears to be a side reaction that is most pronounced in the presence of ATP.

Does the loss of non-native polypeptide occur from the *cis*- or the *trans*-ring of the GroEL–GroES complex, or from both sides? Rhodanese can leave the *cis*-ring of GroEL only when GroES dissociates from GroEL. If the release of all of the non-native protein were to be based on this mechanism, all unfolded rhodanese should be

retained on GroEL in the presence of GroES and AMP–PNP, conditions under which GroES does not cycle. As shown in Figure 3C, this hypothesis is incorrect; ~60% of the GroEL-bound rhodanese was released from GroEL in a non-native state upon incubation with GroES and AMP–PNP. This dissociation of rhodanese occurred from the *trans*-ring of GroEL and was unproductive for folding (see Figure 8). If binding of GroES with ATP has the same effect as binding in the presence of AMP–PNP, the efficient inhibition of rhodanese folding by GroEL-trap in reactions that contain ATP would be explained. In each chaperonin cycle, GroES and ATP bind either to the polypeptide-containing ring or to the free ring of GroEL (Hendrick and Hartl, 1995) (see Figure 8). Additionally, release of non-native protein can occur from the *cis*-ring, when GroES dissociates (see below).

A functional single-ring GroEL

The finding of efficient rhodanese folding, mediated by GroES cycling in the presence of ADP, indicated that successive rounds of ATP hydrolysis in the two GroEL rings are not a basic mechanistic requirement of chaperonin function, at least for this substrate protein *in vitro*. If indeed folding depends essentially on the binding and release of GroES from the polypeptide-containing ring of GroEL, conditions may exist in which a single-ring chaperonin can support this reaction. However, a recent study with such a single-ring variant, SR1, suggested that this prediction is incorrect (Weissman *et al.*, 1996). The subunits of SR1 contain four point mutations in amino acid residues Arg452, Glu461, Ser463 and Val464 that make the major contacts between the two rings of GroEL (Weissman *et al.*, 1995). Residue 452 is replaced by Glu and the other three residues by Ala. These mutations cause SR1 to form single heptameric rings that do not associate to double-rings in the presence of GroES and ATP (Weissman *et al.*, 1996). SR1 has previously been shown to fold rhodanese upon binding of GroES; however, the folded protein was not released from the ring cavity because GroES did not dissociate (Weissman *et al.*, 1996). It was concluded that a round of ATP hydrolysis in the opposite GroEL ring would be required to drive the dissociation of GroES and thus the release of folded protein. This seemed surprising in light of our finding that the presence of rhodanese itself can induce GroES cycling from GroEL, independent of ATP hydrolysis. In search of an explanation for this discrepancy, we generated the SR1 mutant and purified the protein upon overexpression in *Escherichia coli*.

As shown in Figure 6A, the SR1–rhodanese complex fractionated as a single ring upon size-exclusion chromatography. In the presence of ATP and GroES, SR1 supported the folding of rhodanese at a rate and yield close to that of GroEL [Figure 6B, panel (i)]. Interestingly, folding by SR1 in the presence of ADP was almost as fast as in the presence of ATP, in contrast to the observations made with GroEL [Figure 6B, panel (ii)]. This suggests that the free single ring differs structurally and functionally from normal GroEL rings that are connected. We next asked whether GroES cycled between SR1-bound and free states under these conditions, by measuring the exchangeability of SR1-bound [^{3}H]GroES (see Figure 4). GroES exchange (expressed as the amount of SR1-bound [^{3}H]GroES after

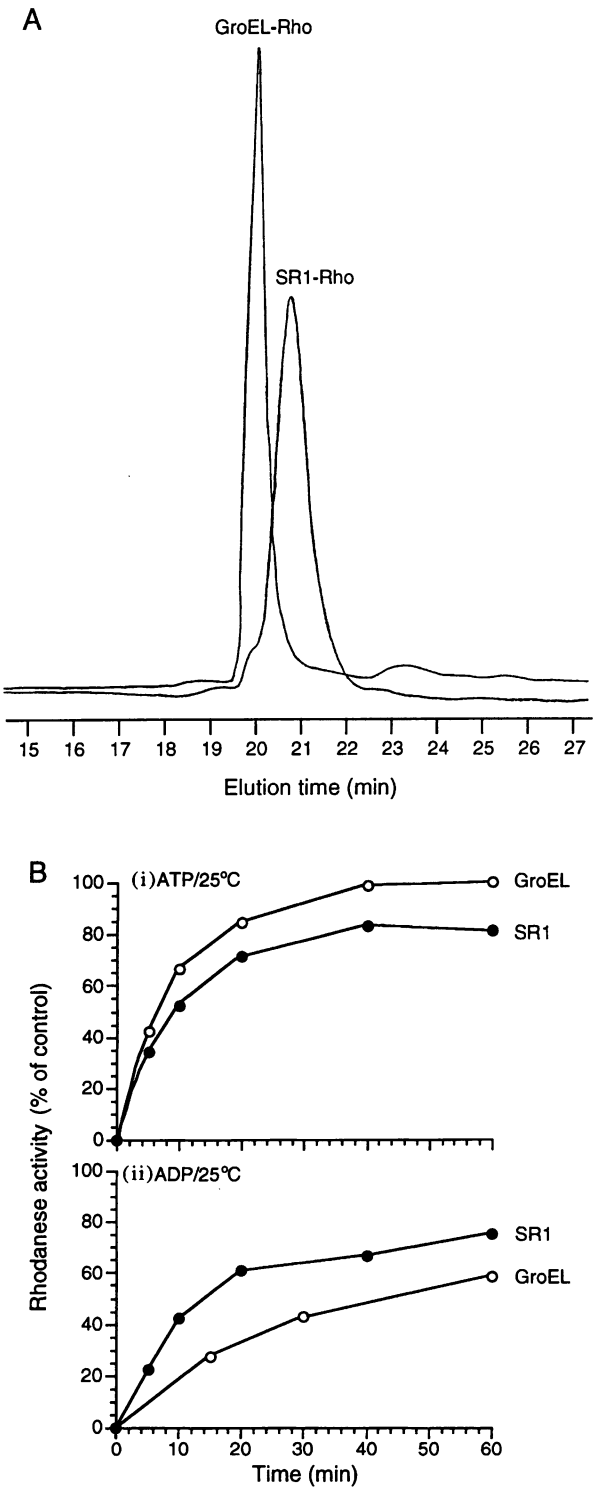

Fig. 6. Nucleotide and GroES-dependent reactivation of rhodanese bound to GroEL or SR1. (**A**) Gel filtration analysis of GroEL–rhodanese and SR1–rhodanese complexes. Approximately equal protein amounts of the isolated complexes (~0.8 mg) were applied to a TSK G3000SW column equilibrated in 20 mM MOPs/KOH, pH 7.2, 100 mM KCl (flow rate 0.5 ml/min). Note that the SR1 preparation contains a small contamination by GroEL (~4%), visible as a shoulder at 20 min elution time. (**B**) Isolated GroEL or SR1 rhodanese complexes (1 μM each) were incubated with 2 μM GroES at 25°C with (i) 5 mM ATP and (ii) 0.2 mM ADP (GroEL, ○; SR1, ●). Folding was initiated by addition of the respective nucleotide. At the times indicated, rhodanese activities were determined for 10 min at 37°C.

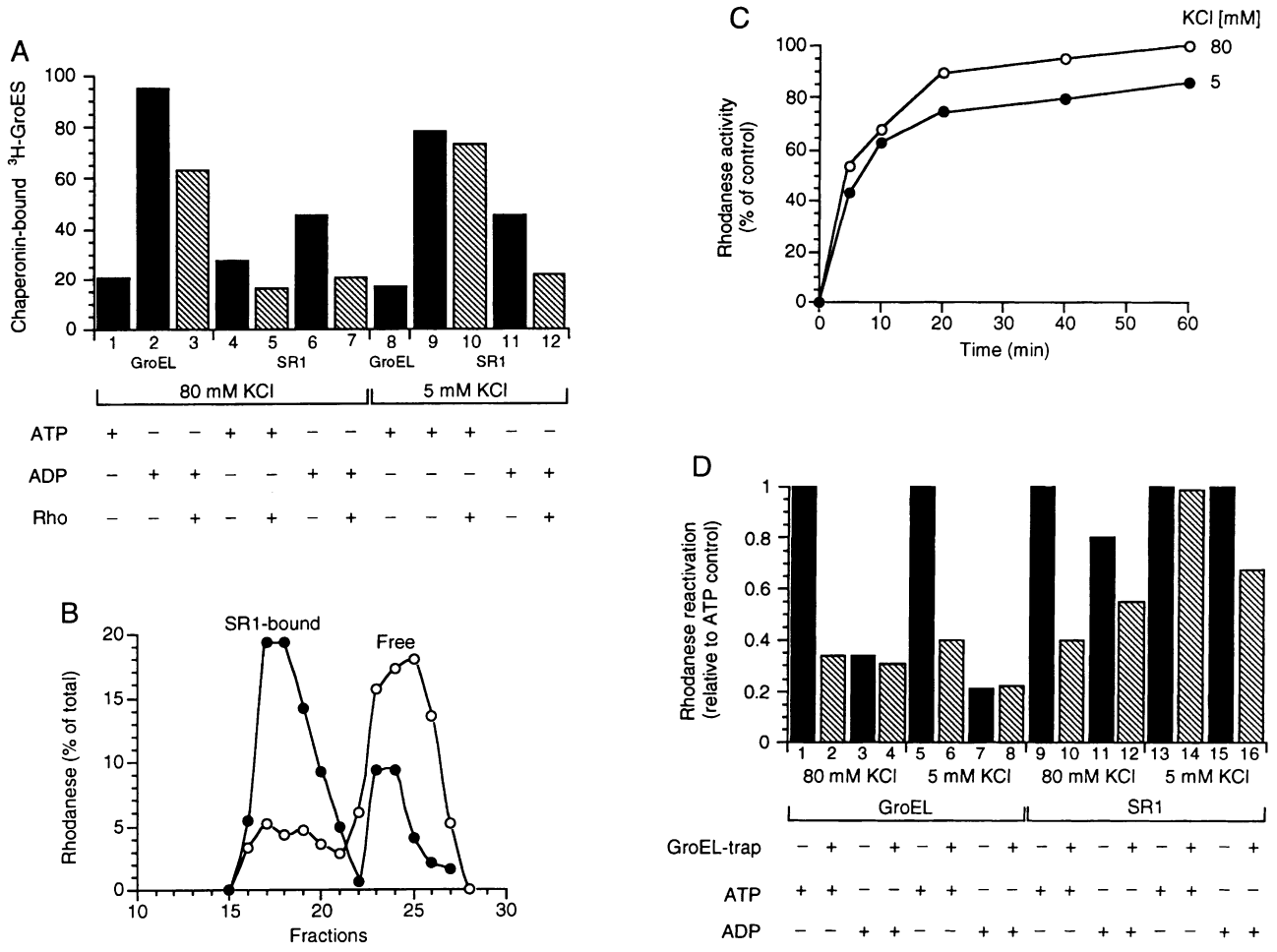

Fig. 7. Comparative analysis of GroEL–rhodanese and SR1–rhodanese complexes with respect of GroES cycling and rhodanese release. (**A**) Exchange of chaperonin-bound [^{3}H]GroES by unlabelled GroES in high K^+ (80 mM KCl) (reactions 1–7) and low K^+ (5 mM KCl) buffer (reactions 8–12). GroEL or SR1 (1 μM) with or without bound rhodanese was incubated with 1 μM [^{3}H]GroES in buffer A/5 mM Mg-acetate/20 mM sodium thiosulfate or buffer B/12 mM Mg-acetate/20 mM sodium thiosulfate (Weissman *et al.*, 1996) in the presence of 5 mM ATP or 0.2 mM ADP at 25°C. A 5-fold excess of unlabelled GroES over [^{3}H]GroES was added and at 10 min GroES exchange was stopped by the addition of 30 mM glutaraldehyde (see Figure 4 and Materials and methods). Reactions were analyzed by native PAGE, autoradiography and densitometry. Amounts of GroEL-bound [^{3}H]GroES in the absence of unlabelled GroES are set to 100%. (**B**) Release of rhodanese from SR1. GroES (5 μM) was added to an isolated SR1–rhodanese complex (2.5 μM) in buffer A/5 mM Mg-acetate/20 mM sodium thiosulfate (○) or buffer B/12 mM Mg-acetate/20 mM sodium thiosulfate (●) and refolding was initiated by the addition of 5 mM ATP. After 20 min at 25°C, rhodanese activities were determined in the absence of CDTA and the same aliquots used in the enzyme reactions were then separated on a Sepharcryl S300-HR gel filtration column equilibrated in buffer A/5 mM Mg-acetate/20 mM sodium thiosulfate/5 mM ATP or buffer B/12 mM Mg-acetate/20 mM sodium thiosulfate/5 mM ATP. Fractions of 0.5 ml were TCA precipitated and analyzed by SDS–PAGE and immunoblotting with rhodanese antibodies, followed by densitometry. The peak of SR1 eluted after ~45 min in fraction 18 (GroEL eluted at fraction 12; see Figure 3). Amounts of rhodanese are given in percentage of the total eluted from the column. The recovery was ~85%. (**C**) Isolated SR1 rhodanese complexes (1 μM) in buffer A/5 mM Mg-acetate/20 mM sodium thiosulfate or buffer B/12 mM Mg-acetate/20 mM sodium thiosulfate were incubated with 2 μM GroES at 25°C with 5 mM ATP (80 mM KCl, ○; 5 mM KCl, ●). Folding was initiated by addition of nucleotide. At the times indicated, rhodanese activities were determined. The maximal rhodanese activity reached in the presence of 80 mM KCl was set to 100%. (**D**) GroEL and SR1–rhodanese complex (1 μM) was incubated at 25°C in buffer A/5 mM Mg-acetate/20 mM sodium thiosulfate (columns 1–4 and 9–12) or buffer B/12 mM Mg-acetate/20 mM sodium thiosulfate (columns 5–8 and 13–16) either in the absence (solid bars) or presence (hatched bars) of a 5-fold molar excess of GroEL-trap (M-GroEL) over chaperonin. Refolding was initiated by the addition of 5 mM ATP or 0.2 mM ADP. Rhodanese activities were determined after 20 min. The activity measured in the presence of 5 mM ATP and absence of GroEL-trap was set to 100%.

incubation with excess unlabelled GroES) was indeed observed in the presence of both ATP and ADP (Figure 7A, columns 4–7 and 9–12). Exchange in the presence of ADP was significantly accelerated by bound rhodanese (Figure 7A, columns 6, 7 and 11, 12). These results differed from the observations by Weissman *et al.* (1996) who had reported that GroES remains bound to GroEL, even in the presence of substrate protein. This discrepancy resulted because these investigators used a low-salt buffer containing 5 mM KCl, whereas our buffer solution included 80 mM KCl. A comparison confirmed that at 5 mM KCl, in the presence of ATP, most SR1-bound GroES was non-exchangeable, irrespective of the association of rhodanese (Figure 7A, columns 9, 10). Surprisingly, we detected significant cycling of GroES from SR1 with ADP, even at 5 mM KCl and in the absence of rhodanese (Figure 7A, columns 11, 12). The ATPase activity of GroEL is K^+-dependent, 5 mM K^+ being sufficient for optimal ATP hydrolysis (Todd *et al.*, 1993) and efficient GroES cycling (Hayer-Hartl *et al.*, 1995) (Figure 7A, column 8). Indeed, exchange of SR1-bound GroES was also observed in a buffer containing 5 mM K^+ and 80 mM

Na^+ (not shown), confirming the notion that the SR1–GroES complex is salt-sensitive (Weissman *et al.*, 1996). Under the conditions of GroES cycling, ATP hydrolysis of SR1 was only partially inhibited by GroES, whereas complete inhibition was observed under the low-salt conditions (not shown), presumably due to arresting SR1 in an ADP-bound state (Weissman *et al.*, 1995). This stable SR1–ADP–GroES state seems to be formed only through ATP hydrolysis and not by direct binding of ADP, as suggested by the finding that GroES bound to SR1 tightly upon incubation with ATP but not ADP (Figure 7A, columns 9, 10 and 11, 12). Such a difference in GroES binding in the presence of ATP and ADP is not observed with GroEL (Martin *et al.*, 1993; Todd *et al.*, 1993).

Release of native and non-native rhodanese from SR1

Under conditions of GroES cycling (80 mM KCl in the presence of ATP or ADP) folded rhodanese was efficiently released from SR1. This was demonstrated by size-exclusion chromatography of a preformed SR1–rhodanese complex after incubation with ATP and GroES (Figure 7B). In contrast, only ~20–30% of the bound rhodanese was released from SR1 under conditions that prevented efficient GroES release (5 mM KCl, presence of ATP). Surprisingly, the rate and overall yield of rhodanese folding was essentially the same, whether GroES cycled or not (Figure 7C). This is important because GroES binding and release is generally thought to be coupled to rounds of folding and unfolding of kinetically trapped intermediates. Since kinetically trapped folding intermediates are apparently generated during rhodanese folding (Figure 3C; see also Mendoza *et al.*, 1991), our results suggest that their unfolding or rearrangement by SR1 requires neither ATP hydrolysis nor the (complete) release and rebinding of GroES. This may be due to an increased conformational flexibility of SR1 as compared with GroEL.

Having defined the conditions that allowed us to examine the function of SR1 with and without GroES cycling, we analyzed the folded state in which rhodanese leaves SR1 upon GroES dissociation. GroEL or SR1 with bound rhodanese was incubated with or without a 5-fold molar excess of GroEL-trap (Figure 7D). Under conditions of GroES cycling with ATP (80 mM KCl), GroEL-trap inhibited the folding of rhodanese from SR1 with similar efficiency than that from GroEL, indicating that a significant fraction of rhodanese left the chaperonin in a non-native state (Figure 7D, columns 1, 2 and 9, 10). However, at 5 mM K^+ in the presence of ATP only GroEL-mediated folding was inhibited by GroEL-trap, whereas the renaturation of SR1-bound rhodanese was unimpaired (Figure 7D, columns 5, 6 and 13, 14). This lack of inhibition was expected, because GroES remained stably bound to SR1 under the low-salt conditions, preventing the premature exit of substrate from the folding cage. Interestingly, little inhibition by GroEL-trap was also observed with SR1 in the presence of ADP, where GroES cycled efficiently (Figure 7D, columns 15, 16). Thus, incompletely folded polypeptide present in the cavity may reassociate with the chaperonin without entering into the bulk solution upon GroES release. We suggest that the extent to which non-native protein rebinds or exits the GroEL cavity may not only depend on the rate of GroES cycling, but also on the conformation of the apical GroEL domains at the time of GroES dissociation.

Discussion

GroEL/GroES-mediated folding of rhodanese was dissected into subreactions that help to define the basic mechanism of chaperonin action. In support of the view that GroEL is essentially a folding cage (Creighton, 1991; Agard, 1993; Martin *et al.*, 1993; Ellis, 1994a, 1996; Corrales and Fersht, 1995), the minimal requirement for the folding of rhodanese is the binding of GroES to the polypeptide-containing ring of GroEL, causing displacement of bound rhodanese from the hydrophobic surface of the GroEL cavity into its sequestered volume (Figure 8). When GroES dissociates, thereby opening the GroEL cage, folded protein is released into the bulk solution. Significantly, this cycling of GroES can be induced by the substrate protein itself, independent of ATP hydrolysis by the chaperonin. This is possible with both wild-type GroEL and with a single-ring variant of GroEL. Thus, our observations suggest a re-evaluation of the role of ATP hydrolysis in the chaperonin mechanism. Efficient ADP- and GroES-dependent folding of rhodanese has also been observed by Fisher and colleagues (M.Fisher, personal communication).

In current models of chaperonin action, GroES binding and release is driven by successive rounds of ATP hydrolysis in the two GroEL rings (Jackson *et al.*, 1993; Martin *et al.*, 1993; Todd *et al.*, 1993, 1994; Burston *et al.*, 1995; Hayer-Hartl *et al.*, 1995; Corrales and Fersht, 1996). Figure 8A (reaction 1) shows such a model, along with the experimentally resolved subreactions of the chaperonin mechanism. Binding of seven ATPs and of GroES to the polypeptide-containing ring of GroEL (i.e. in *cis*) discharges the polypeptide from the hydrophobic binding regions of the apical GroEL domains and initiates folding in the cavity. Hydrolysis of ATP generates a stable GroEL–7ADP–GroES complex that encloses the polypeptide. This complex dissociates upon a subsequent round of ATP hydrolysis in the opposite GroEL ring. In contrast, binding of GroES and substrate in *trans*-position is unproductive for folding (Figure 8A, reaction 1′; and figure legend).

Notably, this GroEL–GroES ATPase cycle has largely been defined in the absence of substrate protein, based on the assumption that unfolded polypeptide does not affect GroES binding and release (Todd *et al.*, 1994). While probably correct for small substrate proteins, such as barnase (~12 kDa), this assumption does not hold for the larger polypeptide rhodanese (~33 kDa), at least at physiologically relevant salt concentrations. The presence of rhodanese in the GroEL cavity destabilizes the GroEL–7ADP–GroES complex, presumably due to substrate stimulating the dissociation of bound ADP (Martin *et al.*, 1993; Hayer-Hartl *et al.*, 1995) (Figure 8A, reaction 2). This interplay between substrate, GroEL and GroES results in efficient GroES cycling in the presence of ADP alone, in contrast to the observations with AMP–PNP (Figure 8A, reaction 3). Thus, in the presence of ADP, the interconversion of the high- and low-affinity states of GroEL for substrate becomes independent of both ATP

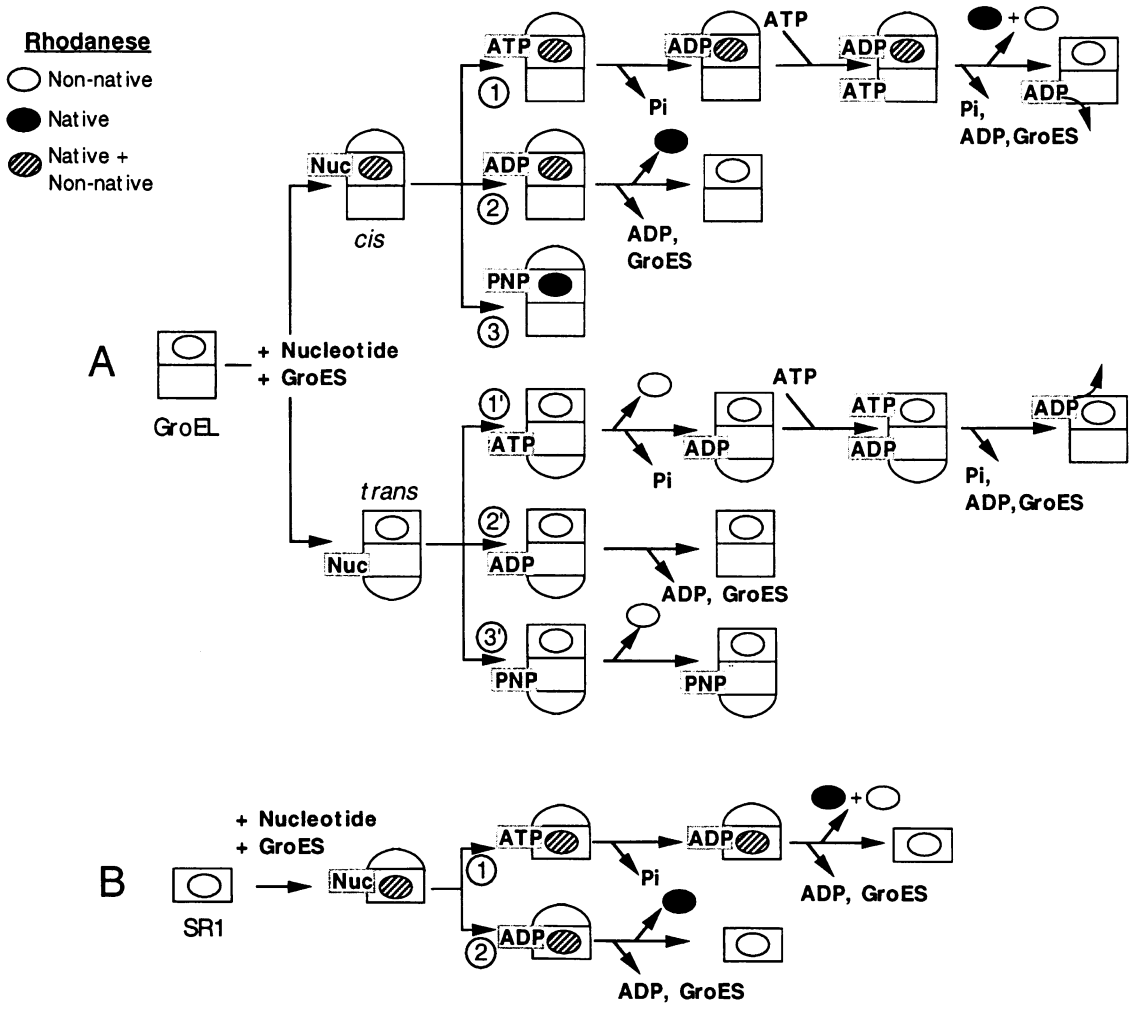

Fig. 8. Experimentally resolved subreactions in the chaperonin-mediated folding of rhodanese. (**A** and **B**) Models for the folding by wild-type GroEL and by the single-ring variant of GroEL, SR1, in the presence of ATP (1), as well as ADP (2) and AMP–PNP (3). Binding and dissociation of nucleotides is thought to occur cooperatively to the seven subunits of a GroEL ring. In reactions (1) and (2) binding of GroES to the polypeptide-containing ring initiates folding in the GroEL cavity, but not all enclosed protein reaches the native state before GroES is released. As a consequence, some non-native rhodanese may leave in addition to native protein (1) [not shown in (2), because this effect is less pronounced with ADP]. Non-native protein is either retained or rebinds to chaperonin, resulting in the rearrangement of kinetically trapped intermediates in preparation for another cycle of GroES-dependent folding. In reactions (1′)–(3′) GroES binds to the GroEL ring in *trans* to rhodanese. This results in release of non-native protein (1′) and (3′) [not observed in (2′)], which will rebind to chaperonin. Folding of this protein is achieved when GroES rebinds in *cis*. In reactions (1) and (1′) a second GroES may bind to GroEL before GroES dissociates from the *cis*-ring. However, this is not an essential step of the reaction (Mayhew *et al.*, 1996; Weisssman *et al.*, 1996) and is therefore omitted from the diagram.

hydrolysis and the negative allosteric coupling of the two GroEL rings. Consistent with this, folding is efficiently supported by a single ring of GroEL (Figure 8B). The participation of symmetrical GroEL–$GroES_2$ complexes in this basic mechanism can be excluded, because these complexes do not form in the presence of ADP (Langer *et al.*, 1992; Azem *et al.*, 1994; Llorca *et al.*, 1994; Schmidt *et al.*, 1994a; Engel *et al.*, 1995).

The ability to separate the ATPase function of GroEL from its protein-folding function provided insight into the mechanistic significance of 'quantized' ATP hydrolysis by GroEL, i.e. the cooperative hydrolysis of units of 7ATP. It has been proposed that GroEL mediates protein folding by utilizing the energy of quantized ATP hydrolysis to unfold kinetically trapped folding intermediates (Todd *et al.*, 1994). Unfolded protein is then released into the bulk solution where it either folds spontaneously to the native state or re-binds to GroEL for unfolding in preparation for another folding trial. The requirement for GroES in this 'iterative annealing' model (Todd *et al.*, 1996) arises from the capacity of GroES to increase the cooperativity of the GroEL ATPase (Gray and Fersht, 1991), thereby rendering ATP hydrolysis quantized (Todd *et al.*, 1994, 1996). Our present and previous studies (Mayhew *et al.*, 1996) have put this model to a test and suggest that: (i) unfolding steps in GroEL-assisted protein folding are not directly driven by the energy from ATP hydrolysis by GroEL; and (ii) that the observed release of unfolded protein from GroEL into the bulk solution does not produce correctly folded protein in the case of GroES-dependent substrates. Rather than increasing the cooperativity of the GroEL ATPase, the critical function of GroES is to displace the substrate protein into an enclosed folding compartment.

In contrast to models in which GroEL and GroES function mainly as an unfoldase (Todd *et al.*, 1994, 1996;

Weissman *et al.*, 1994), we and others have presented evidence that both protein folding and unfolding/rearrangement occur in the sequestrated environment of the GroEL cavity in iterative cycles (see Figure 8) (Corrales and Fersht, 1996; Mayhew *et al.*, 1996). In this model, unfolding steps are the result of the hydrophobic interaction between kinetically trapped folding intermediates and the binding surfaces of GroEL. Our conclusion that ATP hydrolysis is not required for unfolding is further supported by findings with another GroES-dependent substrate protein, malate dehydrogenase (MDH), which forms a kinetically trapped folding intermediate at 30°C that can be rescued by GroEL and GroES (Ranson *et al.*, 1995). MDH folding by GroEL at 30°C is possible in the presence of GroES and ADP (K.Ewalt and M.K.Hayer-Hartl, unpublished results; M.Fisher, personal communication). In contrast to our previous assumption, complete GroES release is apparently not necessary to allow the rearrangement of unproductive protein structures by GroEL. This is suggested by the finding that rhodanese folded under conditions in which GroEL-bound GroES did not exchange with free GroES. We assume that, upon GroES binding, some interaction between the substrate protein and the surface of the GroEL cavity can still occur. This may allow unproductive folding intermediates to overcome kinetic barriers, even under conditions where GroES maintains its contact with GroEL.

What then is the role of ATP hydrolysis by GroEL? It must be emphasized that our present study defines a minimal mechanism of chaperonin action *in vitro*. Clearly, folding occurred at a reduced rate under most conditions where GroES cycling was slow. We propose therefore that ATP hydrolysis by GroEL serves to optimize the rate of the reaction by accelerating GroES cycling and by providing a timing mechanism for GroES binding and release that is presumably adjusted to the rate of folding of most authentic GroEL substrates. Furthermore, ATP hydrolysis-mediated GroES release, and hence two GroEL rings, may be essential for the folding of proteins which cannot induce efficient GroES cycling. The capacity of a substrate to induce cycling may increase with its size, but other structural properties may also be important.

GroES-dependent substrate proteins fold in the GroES-enclosed cavity of GroEL (this study and Mayhew *et al.*, 1996; Weissman *et al.*, 1996). The previously observed release of non-native and non-committed folding intermediates from GroEL into bulk solution is unproductive for folding and may occur when GroES and substrate interact with opposite GroEL rings. In the case of rhodanese, this can be observed upon GroES binding in the presence of ATP or AMP–PNP (Figure 8A, reactions 1′ and 3′) and is apparently a result of the allosteric coupling of the two GroEL rings. Additionally, non-native protein may leave GroEL when GroES dissociates from the polypeptide-containing ring (Figure 8A, reaction 1 and Figure 8B). However, in each reaction cycle only a fraction of the total GroEL-associated protein leaves GroEL prematurely by these routes (Mayhew *et al.*, 1996). The significance of non-native protein release *in vivo* remains to be determined (Ellis and Hartl, 1996).

Materials and methods

Proteins

GroEL was prepared to >98% purity using a GroE-overproducing strain of *E.coli* harboring the plasmid pOF39 (Fayet *et al.*, 1986; Hayer-Hartl *et al.*, 1994). The GroEL-mutant GroEL337/349 (M-GroEL), used as a GroEL-trap, was constructed from the GroEL gene and overproduced as described (Weissman *et al.*, 1994; Mayhew *et al.*, 1996). The single-ring variant of GroEL, SR1, was produced by oligonucleotide-directed mutagenesis of an expression plasmid bearing the gene encoding wild-type GroEL (Weissman *et al.*, 1995).

Glutaraldehyde-crosslinked GroEL (GA-GroEL), also used as a GroEL-trap (Mayhew *et al.*, 1996), was prepared by incubation of 10.6 μM GroEL 14-mer in buffer A (20 mM MOPS–KOH, pH 7.2, 80 mM KCl, 20 mM NaCl) with 0.15% glutaraldehyde for 60 min at 0°C. The reaction was stopped by addition of 300 mM Tris, pH 8.0. GA-GroEL was isolated on a G25 column (PD10, Pharmacia) equilibrated in buffer A and concentrated using Centriprep 100 (Amicon).

GroES was expressed from a pET-11a vector (Novagen Inc.) (a kind gift from Dr J.Flanagan) in BL21 cells cultured at 37°C in LB medium containing ampicillin. Expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 0.8 mM at an OD_{600} of 0.6 and the cells were further incubated for 3 h. GroES was purified as described (Hayer-Hartl *et al.*, 1994). Fully functional [^{3}H]GroES was prepared according to Langer *et al.* (1992). Bovine rhodanese was prepared and ^{3}H-labelled as described (Hlodan *et al.*, 1995).

Protein concentrations were determined based on quantitative amino acid analysis and the colorimetric protein assay of Bradford (1976).

GroEL–rhodanese complexes and rhodanese refolding

Rhodanese was denatured in 6 M guanidinium hydrochloride as described by Langer *et al.* (1992) and diluted 100-fold into buffer A/5 mM Mg-acetate or buffer B (20 mM MOPS–NaOH, pH 7.2, 5 mM KCl) containing 4–6 μM GroEL 14-mer or SR1 7-mer. Protein aggregates were removed by centrifugation at 10 000 *g* for 15 min at 25°C. The supernatant was applied to a 30×1 cm gel filtration column (Sepharcryl S300-HR, Pharmacia) equilibrated in buffer A/5 mM Mg-acetate or buffer B/12 mM Mg-acetate. The GroEL–rhodanese or SR1–rhodanese peak fractions were combined and concentrated on Centriprep 100 (Amicon).

Rhodanese enzyme activities were determined according to Martin *et al.* (1991) and Tandon and Horowitz (1986) with modifications as specified in the figure legends. Unless stated otherwise, the assay mixture contained 15 mM *trans*-1,2-diaminocyclohexane-N,N,N′,N′-tetra-acetic acid (CDTA) to stop GroEL–GroES-mediated rhodanese refolding.

Analysis of GroES exchange

GroEL or SR1 (1 μM) with or without bound rhodanese was incubated in buffer A, 5 mM Mg-acetate or buffer B, 12 mM Mg-acetate (Weissman *et al.*, 1996) with 1 μM [^{3}H]GroES in the presence of nucleotide as specified in the figure legends and incubated for 5 min at 25°C or 37°C. A 5-fold excess of unlabelled GroES over [^{3}H]GroES was added and at various times GroES exchange was stopped by the addition of 30 mM glutaraldehyde (GA) for 1 min. GA action was quenched by 100 mM Tris, pH 8.0, containing 10 mM CDTA. Samples were then applied to G-25 spin columns equilibrated in 10 mM Tris, pH 7.6, 80 mM KCl, 20 mM NaCl, 5 mM EDTA. Reactions were analyzed by native PAGE and fluorography. Amounts of GroEL-bound [^{3}H]GroES in the absence of unlabelled GroES are set to 100%, typically corresponding to one GroES 7-mer bound per GroEL 14-mer or SR1 7-mer.

Ion exchange chromatography of nucleotides

The nucleotides investigated were ATP (Sigma, A-2383), ADP (1) (Sigma, A-2754), ADP (2) (Boehringer, 236675), AMP–PNP (Boehringer, 102547) and AMP (Sigma, A-4659). 200 ml of a 0.85 mM solution of the respective nucleotide in 100 mM NH_4HCO_3 was applied to a Pharmacia HR5/5 Mono-Q column at 4°C at a flow rate of 1 ml/min with a linear gradient of 100–600 mM NH_4HCO_3. The eluted nucleotides were detected by absorbance at 280 nm (also see Horst *et al.*, 1996).

Miscellaneous

The following procedures were carried out according to published methods: native-PAGE (Langer *et al.*, 1992); electrotransfer to nitrocellulose (Towbin *et al.*, 1979); immunolabelling using the luminescence-based detection system ECL (Amersham) (Vachereau, 1989).

Acknowledgements

The authors thank M.Fisher for communicating unpublished observations and B.Netzer for critically reading the manuscript. This work was

supported by the National Institutes of Health and the Howard Hughes Medical Institute. F.W. is a fellow of the Studienstiftung des Deutschen Volkes.

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Received on July 8, 1996; revised on August 8, 1996