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Chaperonin-mediated protein folding: GroES binds to one end of the GroEL cylinder, which accommodates the protein substrate within its central cavity

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The mechanism of GroEL (chaperonin)-mediated protein folding is only partially understood. We have analysed structural and functional properties of the interaction between GroEL and the co-chaperonin GroES. The stoichiometry of the GroEL 14mer and the GroES 7mer in the functional holo-chaperonin is 1:1. GroES protects half of the GroEL subunits from proteolytic truncation of the ~50 C-terminal residues. Removal of this region results in an inhibition of the GroEL ATPase, mimicking the effect of GroES on full-length GroEL. Image analysis of electron micrographs revealed that GroES binding triggers conspicuous conformational changes both in the GroES adjacent end and at the opposite end of the GroEL cylinder. This apparently prohibits the association of a second GroES oligomer. Addition of denatured polypeptide leads to the appearance of irregularly shaped, stain-excluding masses within the GroEL double-ring, which are larger with bound alcohol oxidase (75 kDa) than with rhodanese (35 kDa). We conclude that the functional complex of GroEL and GroES is characterized by asymmetrical binding of GroES to one end of the GroEL cylinder and suggest that binding of the substrate protein occurs within the central cavity of GroEL.

Key words: chaperonin/GroEL/GroES/protein folding

Introduction

Molecular chaperones are of general importance for the cellular processes of protein folding and assembly (Ellis, 1987; Ellis and van der Vies, 1991; Gething and Sambrook, 1992). The members of the Hsp60 family of stress proteins (GroEL in *Escherichia coli*; Hsp60 in mitochondria; rubisco subunit-binding protein in chloroplasts) have been shown to mediate the folding of many different proteins *in vivo* and *in vitro* (Lorimer, 1992; Hartl *et al.*, 1992). These so-called chaperonins (Hemmingsen *et al.*, 1988) are high molecular weight complexes with ATPase activity. They consist of 14 ~60 kDa subunits that are arranged in two stacked heptameric rings (Hendrix, 1979; Hohn *et al.*, 1979; Pushkin *et al.*, 1982; Hutchinson *et al.*, 1989; Zwickl *et al.*, 1990). In the presence of Mg-ATP or Mg-ADP, complex formation with a co-chaperonin (GroES in *E.coli*; Hsp10 in mitochondria) is observed. This co-chaperonin protein is a heptameric ring of identical ~10 kDa subunits (Chandrasekhar *et al.*, 1986; Lubben *et al.*, 1990; Hartman *et al.*, 1992). Binding of GroES regulates the ATPase activity of GroEL and is required for the full function of GroEL in protein folding (Chandrasekhar *et al.*, 1986; Viitanen *et al.*, 1990; Martin *et al.*, 1991).

The molecular mechanism of chaperonin action is still poorly understood. Three-dimensional structures of GroEL and GroES are not yet available and structure-function relationships have not yet been defined for these proteins by other methods. Notably, the stoichiometry of the GroEL and GroES oligomers in the functional chaperonin unit, the so-called holo-chaperonin, has not yet been established. The recent observation of a mitochondrial chaperonin, which can be active as a single heptameric ring, may suggest that the GroEL double-ring contains two binding sites for GroES and two for the substrate protein (Viitanen *et al.*, 1992). Folding substrate proteins are indeed bound at a stoichiometry of only one or two per 14mer GroEL complex (Goloubinoff *et al.*, 1989; Martin *et al.*, 1991; Mendoza *et al.*, 1991; Buchner *et al.*, 1991; Badcoe *et al.*, 1991; Zahn and Plückthun, 1992; Bochkareva *et al.*, 1992). The chaperonin particle has the appearance in electron micrographs of a double doughnut with a central hole or channel that has a diameter of ~6 nm (Hutchinson *et al.*, 1989; Zwickl *et al.*, 1990) and it is possible that substrate binding occurs within this central cavity. The properties of GroEL-bound proteins are consistent with those of the collapsed intermediate or molten globule-states observed during folding *in vitro* (Höll-Neugebauer *et al.*, 1991; Martin *et al.*, 1991; Langer *et al.*, 1992; van der Vies *et al.*, 1992). Further acquisition of structure of the intermediates occurs during a process of GroES-regulated, ATP-dependent release of the substrate protein from the chaperonin scaffold.

In this study we demonstrate biochemically and by electron microscopic image analysis that the stoichiometry of the GroEL and GroES oligomers in the functional holo-chaperonin is 1:1. GroES binding occurs asymmetrically to either end-surface of the GroEL cylinder causing a marked structural change at the opposite end of the oligomer. This leads to the prevention of C-terminal truncation by protease in half of the GroEL subunits. The folding substrate protein is apparently accommodated within the central cavity of the GroEL cylinder.

Results

Stoichiometry of GroEL-GroES in the functional holo-chaperonin

The function of GroEL in mediating the refolding of a number of denatured proteins, such as rhodanese, is essentially dependent on the co-chaperonin GroES (for review see Hartl *et al.*, 1992; Lorimer, 1992). In an effort to understand the molecular details of the interaction between GroES and GroEL, we first analysed the stoichiometry of

the two components in the functional holo-chaperonin complex. A binding assay was established based on the separation of the GroEL−GroES complex and free GroEL by native polyacrylamide gel electrophoresis (native PAGE). Purified GroES, which was ^{3}H-labelled by reductive methylation, was used as the ligand. The radiolabelled protein migrated as a single band of ~10 kDa on reducing SDS−PAGE (Figure 1A) and was fully active supporting the GroEL-dependent refolding of denatured rhodanese (Figure 1B). Binding of GroES to GroEL was only observed in the presence of Mg−ADP or Mg−ATP and resulted in a distinct shift in mobility of GroEL to a slower migrating species on native PAGE (Figure 2A). Incubation of GroEL with increasing concentrations of GroES revealed that binding was saturated at a concentration of GroES equimolar to that of GroEL (with respect to the oligomeric complexes) (Figure 2B). Even at a 10-fold molar excess of GroES, no further increase in the amount of bound GroES could be detected under these conditions. Essentially identical binding curves were obtained in the presence of Mg−ATP or Mg−ADP (not shown).

Titrating the GroES concentration in the functional assay of GroEL-dependent rhodanese refolding showed that reactivation reached the maximal yield when GroEL and GroES were present in equimolar concentrations (Figure 1B). To rigorously establish that the 1:1 complex is fully functional, the GroEL−GroES complex was formed in the presence of Mg−ATP at a 10-fold molar excess of GroES. The holo-chaperonin was then isolated by gel filtration (Figure 3A) and the ability of the isolated complex to refold rhodanese was tested with and without additional GroES. As shown in Figure 3B, the presence of an excess of GroES

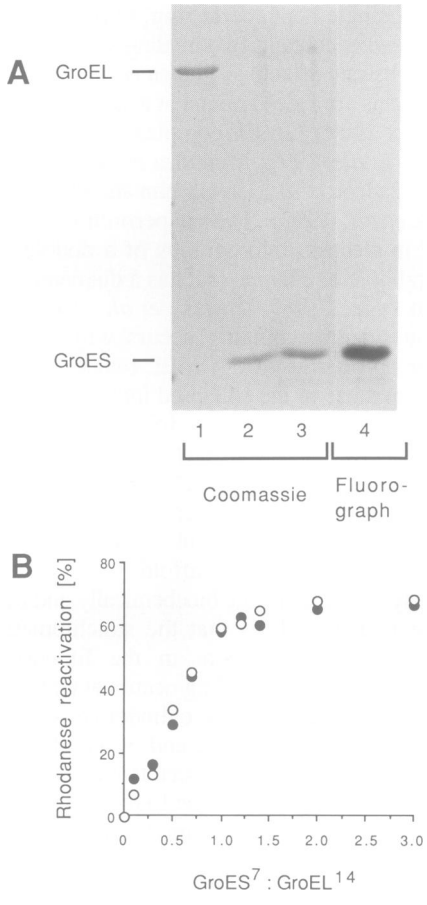

Fig. 1. (**A**) SDS−PAGE of purified GroEL and GroES. Lane 1, GroEL (2 μg); lane 2, GroES (2 μg); lanes 3 and 4, ^{3}H-labelled GroES (3 μg). Lanes 1−3, Coomassie stain; lane 4, fluorograph. (**B**) GroEL-mediated reactivation of denatured rhodanese using ^{3}H-labelled (●) and unlabelled (○) GroES. After binding of guanidinium chloride denatured rhodanese to GroEL oligomer (0.46 μM and 0.69 μM final concentrations, respectively) in buffer A increasing concentrations of GroES were added as indicated and reactivation was started by the addition of 5 mM Mg acetate and 1 mM ATP. The enzymatic activity of rhodanese was determined after incubation for 30 min at 25°C and is expressed as percentage of the activity of the native enzyme control.

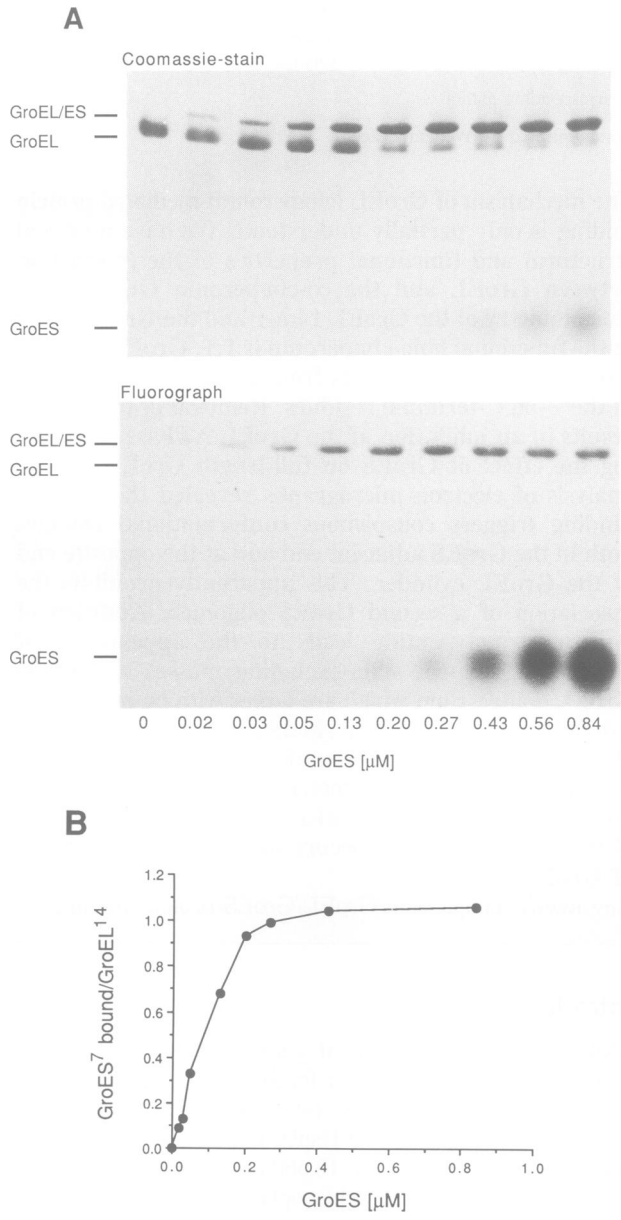

Fig. 2. Complex formation of GroEL and GroES in the presence of Mg−ADP. GroEL oligomer (0.16 μM) was incubated for 10 min at 25°C in buffer A containing 5 mM Mg acetate, 1 mM ADP and ^{3}H-labelled GroES oligomer (0.02−0.84 μM). The GroEL−GroES complex was separated from free GroEL and GroES by native PAGE using a running buffer containing 80 mM MOPS−KOH pH 7.2, 1 mM $MgCl_2$ and 0.1 mM ADP. (**A**) Coomassie-stain and fluorograph; (**B**) quantification of bound GroES by laser densitometry. The positions of the gel of GroEL, GroES and GroEL−ES are indicated.

did not result in an increase of the yield of active rhodanese, thus demonstrating that the isolated 1:1 complex is fully active.

Electron microscopy of GroEL–GroES complexes

The equimolar stoichiometry of GroEL–GroES established biochemically suggested that the holo-chaperonin may have an asymmetrical structure. An electron microscopic analysis of negatively-stained GroEL–GroES complexes was carried out to test this further. The holo-chaperonin was prepared as described above in the presence of Mg–ADP. Figure 4 shows that the preparation almost exclusively contains GroEL–GroES complexes as judged by their characteristic appearance from the side (see below). The individual images of several hundred particles were subjected to image analysis (Figures 5 and 6). As a control, purified GroEL was analysed in parallel in the absence or presence of nucleotides. As previously established, the GroEL oligomer appeared as a cylinder with a diameter of ~14.5 nm and a longitudinal axis of 16 nm. In end-on views the typical 7-fold symmetry of the ring complex is revealed after averaging following rotational and translational alignment via cross-correlation (Figure 5) (Zwickl *et al.*, 1990; Pühler *et al.*, 1992). In the absence of nucleotide the central space of the ring structure had a diameter of ~6 nm (Figure 5A). In the presence of ADP, this central portion of the complex appeared somewhat wider and was more regularly star-shaped (Figure 5C). Global averages of side-views of GroEL showed the four-layered structure that has generally been interpreted as representing the two stacked heptameric rings (Figure 5B and D) (Hutchinson *et al.*, 1989; Zwickl *et al.*, 1990). The GroEL subunits are assumed to be dumb-bell shaped, the two domains corresponding to the two stripes per single ring. No significant Mg–ADP-dependent changes were observed in side-views.

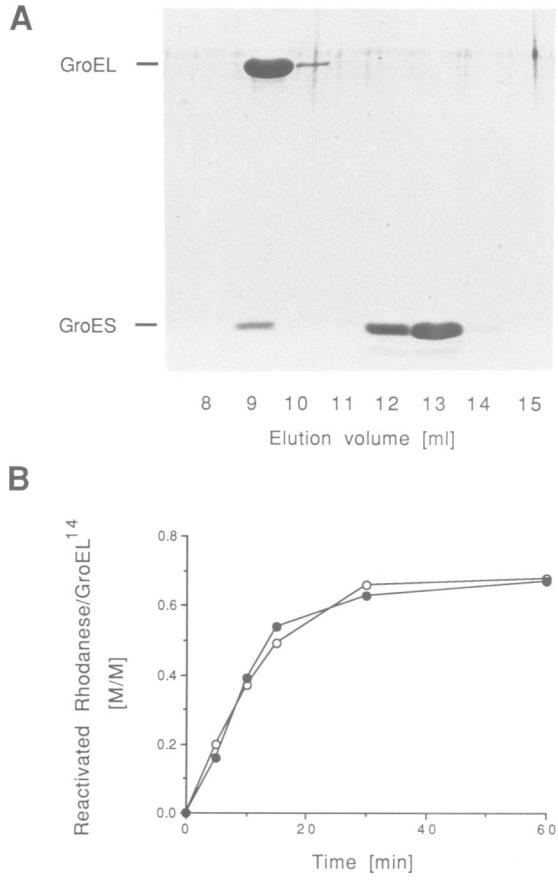

Fig. 3. (**A**) Isolation of GroEL–GroES complex by gel chromatography. GroEL oligomer (0.89 μM) was incubated with 9 μM GroES oligomer in buffer A containing 5 mM Mg acetate and 1 mM ATP. The reaction was passed over a TSK G3000SW column equilibrated with the same buffer (flow rate 0.5 ml/min). Fractions (1 ml) were analysed by SDS–PAGE and Coomassie staining. (**B**) Reactivation of denatured rhodanese by isolated GroEL–GroES complex. GroEL–GroES containing fractions obtained in (A) were pooled and after determination of protein concentration (final concentration of GroEL–GroES ~0.5 μM), divided into halves (○,●). A molar excess of GroES oligomer (1.7 μM, final concentration) was added to one reaction (●) and then denatured rhodanese was added to both reactions (1.4 μM final concentration). Rhodanese activity was determined at the times indicated and is expressed in mol rhodanese reactivated/mol GroEL.

In contrast to GroEL alone, the GroEL–GroES complexes had a markedly different appearance when viewed in the side-on orientation (Figure 6). An additional mass, apparently representing the side-view of a single GroES heptameric ring, which is smaller than a single GroEL heptamer, was detected (arrow). Binding of GroES occurred only to one side of the GroEL cylinder resulting in an asymmetrical shape of the holo-chaperonin. Symmetrical complexes with two bound GroES oligomers per GroEL were not found. Similar observations were made recently with the holo-chaperonin of *Thermus thermophilus* (Ishii *et al.*, 1992). We noted that binding of GroES caused a marked change in the conformation of the GroEL double-toroid. This affected not only the end-layer of GroEL adjacent to GroES, but also the opposite layer of the double-ring. By applying eigenvector–eigenvalue analysis, the data set could be divided into three intrinsically more homogeneous structural classes, in each of which GroEL was altered to different extents, suggesting that the chaperonin has considerable conformational flexibility (Figure 6 A–C). Assuming that the GroEL double-ring is initially symmetrical (see below), pronounced conformational changes would thus occur in equivalent domains of the dumb-bell shaped subunits. The 7-fold rotational symmetry of GroEL was preserved in the complex with GroES as judged from the averaged images of end-on views. Apparently, due to the presence of GroES, the seven centres of mass at the vertices of the polygon, which correspond to the GroEL subunits (see Figure 5A and C), were elongated. The centres of mass of GroES appear to be rotated with respect to those of the GroEL ring by ~15° (Figure 6D).

These experiments confirm the 1:1 stoichiometry of the functional GroEL–GroES complex that was determined biochemically. The marked alteration in conformation of the free end-surface of the GroEL oligomer, which is triggered by the binding of GroES to the opposite end of the cylinder, may explain why GroEL binds only a single GroES oligomer with high affinity.

Protease protection of a 52 kDa fragment of GroEL by GroES

The effects of GroES binding on the conformation of GroEL were further analysed by testing the protease resistance of GroEL. We found that addition of Mg–ATP or Mg–ADP caused a significant stabilization of the GroEL structure towards proteinase K. A protease-resistant fragment of GroEL with an apparent molecular weight of 52 kDa on SDS–PAGE was then produced (Figure 7A). Analysis of

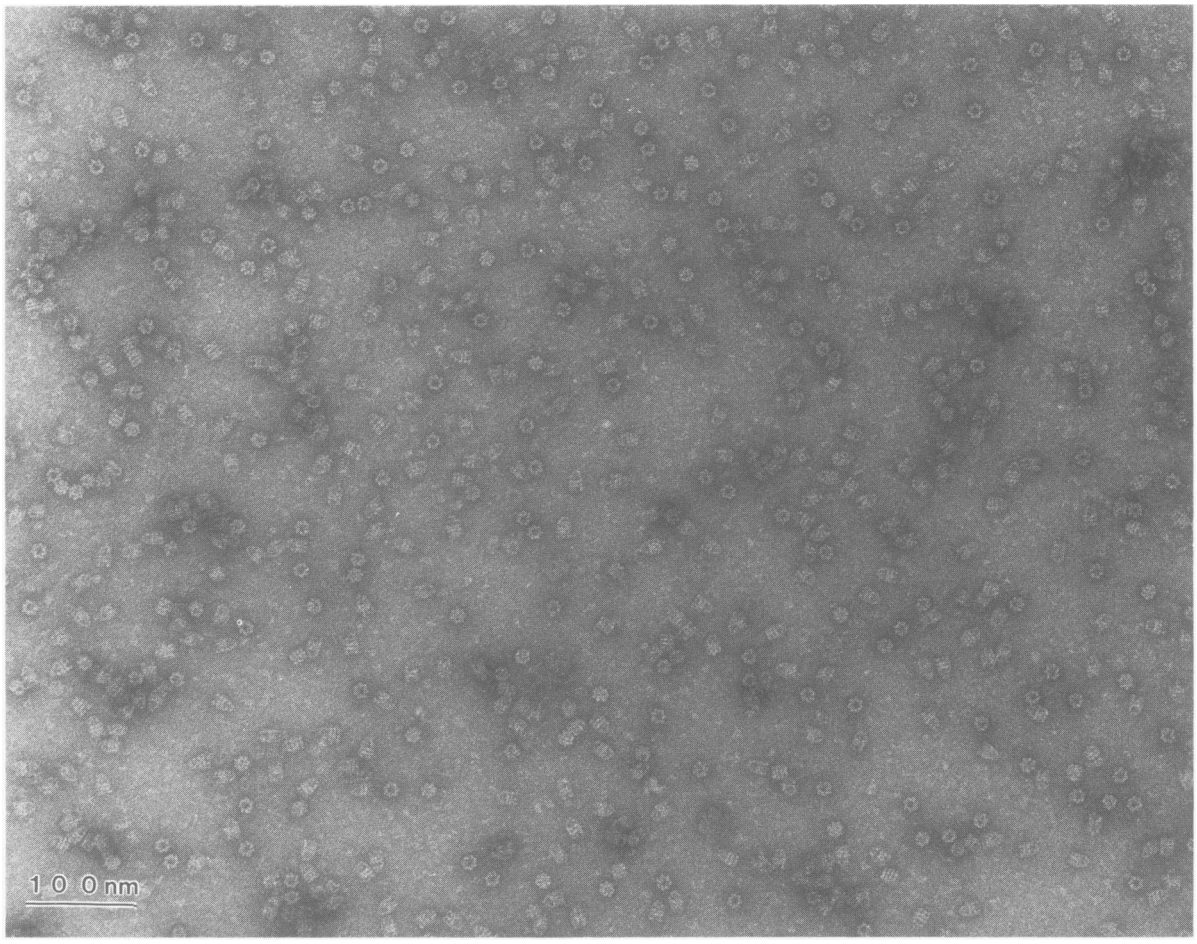

Fig. 4. Electron micrograph of negatively stained GroEL–GroES complexes showing end-on views and side-on views. The GroEL–GroES complex was formed and prepared for electron microscopy as described in Materials and methods.

native PAGE and gel filtration chromatography revealed that the oligomeric structure of the chaperonin was preserved in the truncated GroEL (not shown). Interestingly, upon binding of GroES, protease-treatment resulted in a form of GroEL in which half of the GroEL subunits were protected from degradation as judged by densitometric quantification of Coomassie stained gels. Increasing the concentration of GroES up to a 10-fold molar excess over the GroEL oligomers did not result in the further protection of GroEL subunits (Figure 7B). These findings support the notion that binding of GroES confers asymmetry to the initially symmetrical GroEL double-ring.

N-terminal sequencing revealed that the 52 kDa fragment has the N-terminus of GroEL and thus must be truncated at the C-terminus (GroEL-ΔC). Both GroEL and GroEL-ΔC were subjected to digestion with endolysin C and the resulting peptides were separated by reversed phase HPLC. The peptide patterns of full-length GroEL and GroEL-ΔC differed in two peaks that were absent from the digest of GroEL-ΔC. Both peptides were identified by N-terminal sequencing (not shown) and were shown to correspond to residues 471–498 and 527–548 of the full-length subunit, the latter ending with the C-terminus of GroEL. Consistent with the difference in apparent size between GroEL-ΔC and full-length GroEL on SDS–PAGE, GroEL-ΔC lacks at least the 50 C-terminal residues of GroEL.

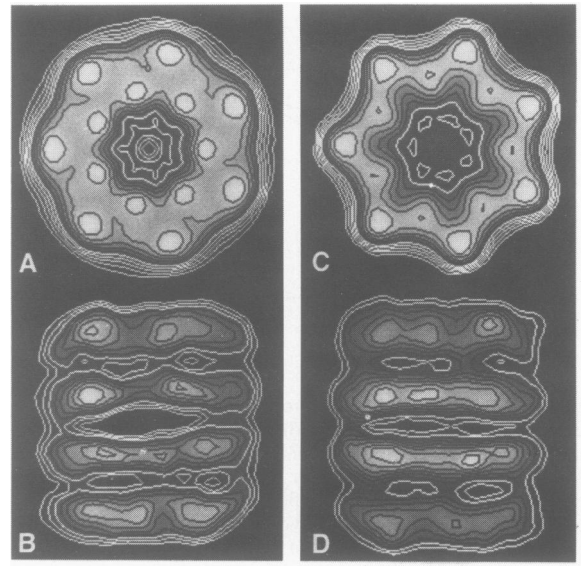

Fig. 5. Averages of end-on and side-on views of electron images of GroEL prepared in the absence (**A** and **B**) and in the presence of Mg–ADP (**C** and **D**). The total number of particles analysed was (A) 360, (B) 270, (C) 900 and (D) 100. The dimensions of the complexes are: A, symmetrized (sym.) diameter 15.6 nm; B, 15.5 × 14.1 nm; C, sym. diameter 16.1 nm; D, 16.0 × 14.4 nm.

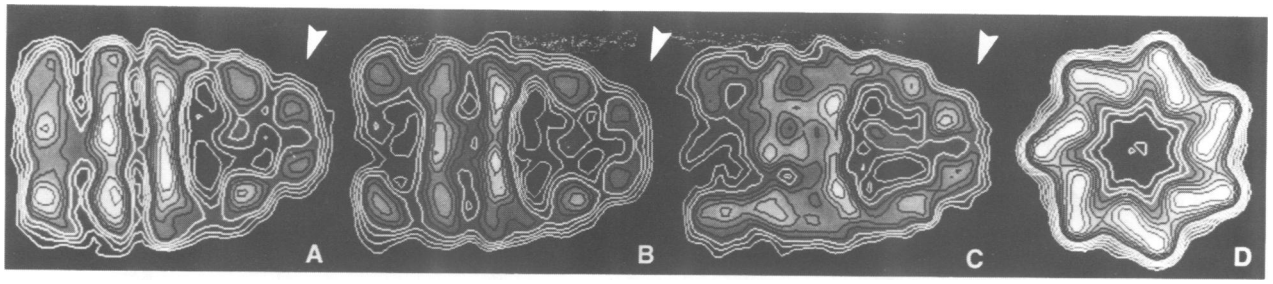

Fig. 6. Averages of electron microscopic images of side-on (**A**–**C**) and end-on views (**D**) of GroEL–GroES complexes shown in Figure 4. (A–C) represent structural classes of particles obtained by eigenvector-eigenvalue data analysis and (**D**) represents the global average of end-on views. The total number of particles analysed was (A) 99, (B) 122, (C) 66 and (D) 343. The dimensions of the complexes are: (A–C), 19.9 × 13.5 nm; (D), sym. diameter 15.4 nm.

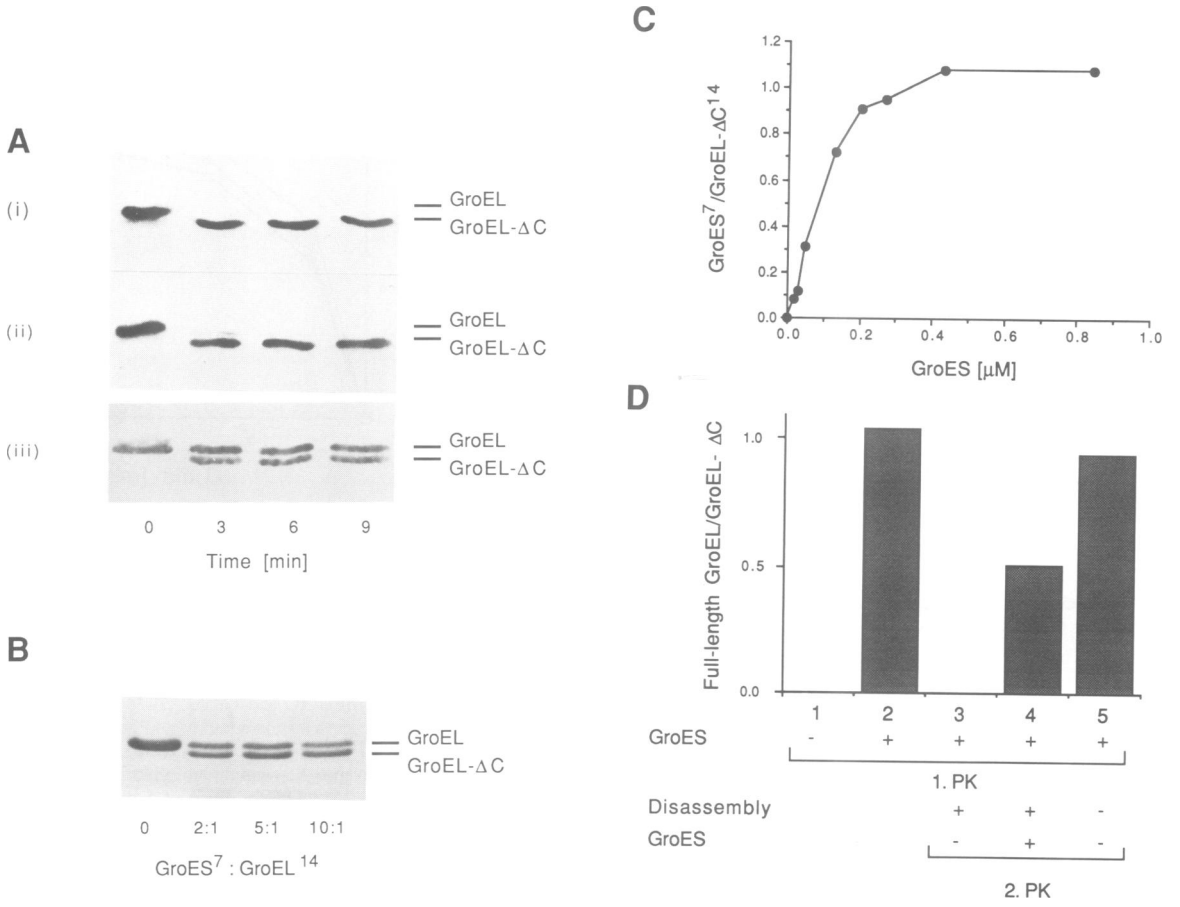

Fig. 7. (**A**) Effect of nucleotides and GroES on the stability of GroEL towards proteinase K. GroEL oligomer (1.25 μM) was incubated for 10 min at 25°C in buffer A containing 5 mM Mg acetate and (i) 1 mM ADP, (ii) 1 mM ATP or (iii) 1 mM ATP and 2.5 μM GroES. The reactions were then cooled to 0°C and incubated in the presence of proteinase K (25 μg/ml final concentration). At the times indicated, aliquots were removed and phenylmethylsulfonylfluoride (PMSF) was added to a final concentration of 1 mM. TCA precipitates were analysed by SDS–PAGE and immunoblotting with anti-groEL antiserum. The positions of GroEL and GroEL–ΔC are indicated. The product of reaction (iii) represents GroEL-ΔC/2. (**B**) GroEL was incubated as in (A) in the presence of 5 mM Mg acetate, 1 mM ATP and 0, 2.5, 7.5 and 12.5 μM GroES oligomer. Protease treatment was performed for 10 min. Samples were analysed by SDS–PAGE. The Coomassie stained gel is shown. (**C**) Binding of ^{3}H-labelled GroES to GroEL-ΔC generated by protease treatment of GroEL for 10 min as in (A). GroEL-ΔC oligomer (0.16 μM) was incubated in buffer A in the presence of 5 mM Mg acetate and 1 mM ADP with increasing concentrations of ^{3}H-labelled GroES as indicated. Complex formation was analysed by native PAGE as described in Figure 2. (**D**) Binding of GroES to GroEL-ΔC/2. GroEL-ΔC/2 was generated as in (A, iii) by protease treatment of GroEL in the absence (lane 1) or presence (lane 2) of GroES and 1 mM Mg ATP. After dissociation of the GroEL–GroES complex in the presence of 5 mM CDTA and reisolation by gel chromatography, GroEL-ΔC/2 was concentrated by ultrafiltration (Centricon-100, Amicon) to a final concentration of 1.25 μM and incubated in buffer A containing 10 mM Mg acetate and 1 mM ADP in the absence (lane 3) or presence of 2.5 μM GroES (lane 4) for 10 min at 25°C. In a control reaction GroEL–GroES complex was re-isolated as described without prior dissociation (lane 5). A second incubation with proteinase K was performed and the reactions were analysed as in (B). Amounts of full-length GroEL and of GroEL-ΔC were quantified by densitometry and are expressed as ratio of full-length GroEL/GroEL-ΔC. PK, proteinase K treatment.

It seemed likely that GroES binding occurs to the C-terminal region of GroEL or close to it, thus directly shielding it from protease. Alternatively, a conformational change of GroEL, more distant to GroES, could lead to the protection of this segment. We found that GroEL-ΔC had retained the ability to bind a single GroES, indicating the

presence of an intact GroES binding site (Figure 7C). This allowed us to address the question whether GroEL-ΔC/2, containing only half of its subunits in the C-terminally truncated form, bound GroES specifically at one of its end-surfaces. GroEL-ΔC/2 was generated first of all by protease treatment of GroEL−GroES complex in the presence of Mg−ATP. Then Mg^{2+} was chelated by adding CDTA and GroEL-ΔC/2 was re-isolated by gel chromatography. This treatment resulted in the dissociation of GroEL-ΔC/2 and GroES. When the free GroEL-ΔC/2 was again incubated with protease (in the presence of Mg−ATP), the remaining full-length GroEL subunits were cleaved to GroEL-ΔC (Figure 7D). In contrast, when GroES was added prior to the second incubation with protease, only about half of the full-length GroEL present in GroEL-ΔC/2 was degraded. These results suggest that GroES is initially free to bind to either end surface of GroEL. Binding induces a conformational change at the opposite surface thereby apparently preventing efficient association of a second GroES oligomer. Our results also indicate that the four stripes in electron microscopic side-views of the GroEL double-ring, which correspond to the two major domains of the stacked subunits, have the orientation A-B B-A rather than A-B A-B.

Functional properties of GroEL-ΔC

Binding of GroES is known to suppress the uncoupled ATPase activity of GroEL measured in the absence of substrate protein (Chandrasekhar *et al.*, 1986; Viitanen *et al.*, 1990; Martin *et al.*, 1991). Interestingly, C-terminal truncation to form GroEL−ΔC resulted in a similar reduction of the ATPase activity of GroEL by 80% (Figure 8A). The same inhibition was observed with GroEL-ΔC/2 that had been dissociated from GroES and re-purified. In a control reaction, GroES inhibited the ATPase of full-length GroEL by 92%. GroES binding to GroEL-ΔC caused a slight further reduction of the ATPase activity (not shown). The addition of α_{s1}-casein, which has the properties of a partially unfolded protein and is known to bind to GroEL (Martin *et al.*, 1991; Langer *et al.*, 1992), stimulated the ATPase of GroEL-ΔC by a similar factor as that of full-length GroEL (Figure 8A). The rate of ATP hydrolysis measured under these conditions was comparable to that seen upon addition of casein to GroEL−GroES (not shown; see Martin *et al.*, 1991). This suggested that cleavage of the C-terminal segment may cause only a limited alteration in the regulation of the GroEL ATPase.

GroEL-ΔC was active in binding denatured rhodanese as demonstrated by native PAGE using the ^{3}H-labelled protein (not shown). Denatured rhodanese, which cannot refold spontaneously except at low temperature or in the presence of detergent (Mendoza *et al.*, 1991), is refolded by GroEL in a process that requires GroES (Martin *et al.*, 1991; Mendoza *et al.*, 1991). We found that both GroEL-ΔC and GroEL-ΔC/2 had maintained the ability of full-length GroEL to refold rhodanese in a GroES and ATP-dependent reaction. Thus, under the experimental conditions *in vitro*, removal of the C-terminal 5 kDa segment causes a pronounced reduction of the uncoupled ATP-hydrolytic activity of GroEL, but does not affect its functional interactions with either GroES or the substrate protein rhodanese. GroEL contains a methionine and glycine-rich sequence at its C-terminus (residues 536−548) that is conserved in several members of the chaperonin family. GroES may exert its inhibitory effect on the GroEL ATPase by directly or indirectly affecting this C-terminal region of GroEL.

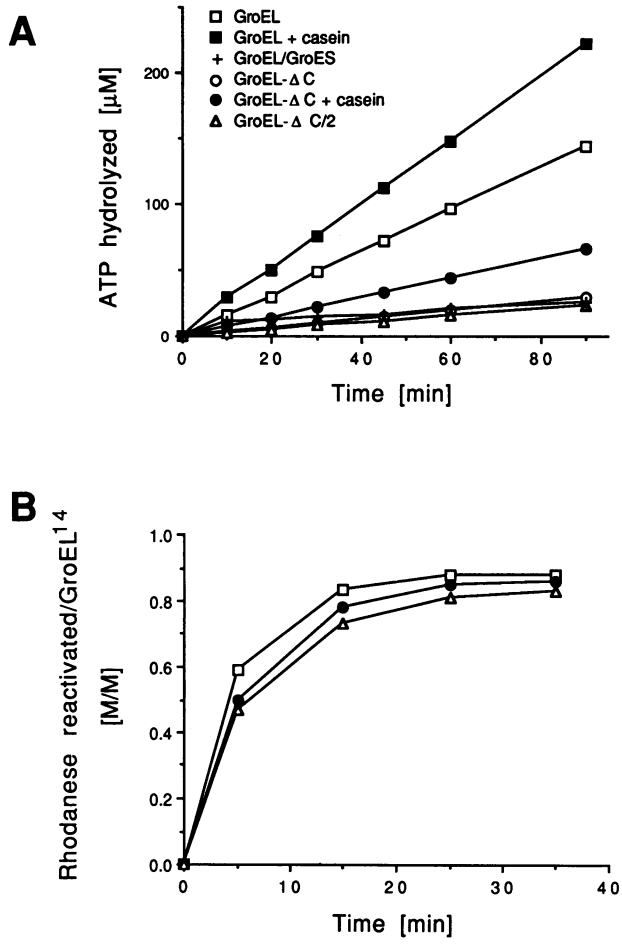

Fig. 8. Functional defects of GroEL-ΔC and GroEL-ΔC/2. (**A**) Effects of GroES and α_{s1}-casein on the ATPase activity of GroEL. 0.17 μM of GroEL oligomer (□,■,+), GroEL-ΔC (○,●) or GroEL-ΔC/2 (Δ) were incubated in buffer A with (■,●) or without (□,○) 0.78 μM casein or with 0.33 μM GroES oligomer (+). Free phosphate was determined colorimetrically (Lanzetta *et al.*, 1978; Lill *et al.*, 1990). (**B**) Reactivation of denatured rhodanese by GroEL, GroEL-ΔC and GroEL-ΔC/2. GroEL-ΔC and GroEL-ΔC/2 generated as in Figure 7 were re-isolated by gel chromatography and adjusted to a concentration of 0.49 μM oligomer. Denatured rhodanese was added by 75-fold dilution from 6 M guanidinium chloride to a final concentration of 0.95 μM. After addition of 5 mM Mg acetate, 1 mM ATP and 1 μM GroES the enzymatic activity of rhodanese was determined at the times indicated. The concentration of reactivated rhodanese was calculated based on the specific activity of the native enzyme.

Electron microscopy of GroEL−substrate protein complexes

The GroEL oligomer binds only one or two molecules of unfolded substrate protein. Several lines of evidence suggest that GroEL stabilizes conformational intermediates that are generated early during folding, which have properties resembling the 'molten globule' state (Martin *et al.*, 1991). Little is known about the topology of the substrate protein at GroEL or its spatial relationship to the symmetrically bound GroES, except that the bound protein is accessible to proteases such as proteinase K (Ostermann *et al.*, 1989;

Martin *et al.*, 1991). To address these questions, we analysed the electron microscopic images of complexes of GroEL and the substrate protein rhodanese. Denatured rhodanese (35 kDa) was bound to GroEL by dilution of the unfolded protein from 6 M guanidinium chloride into a solution containing GroEL. A 3-fold molar excess of rhodanese over GroEL 14mer was used in these experiments, which results in the saturation of GroEL with substrate (not shown). After removal of unbound, aggregated rhodanese by centrifugation, the GroEL–rhodanese complex was isolated by gel filtration chromatography (not shown) and analysed by negative-stain electron microscopy (Figure 9A). Averaging of end-on views revealed an additional stain-excluding mass in the central portion of the GroEL complex. Such stain-excluding masses were never detected when GroEL was analysed in the absence of substrate protein (see Figure 5). The dimensions of the additional density in the centre of GroEL would be compatible with the dimensions of a partially folded, yet compact conformational intermediate of rhodanese stabilized by GroEL. In the presence of substrate protein, the inner circumference of the ring complex showed a deviation from the perfect 7-fold symmetry seen with free GroEL. In contrast, the outer circumference and the diameter of the GroEL cylinder were not significantly different from that of free GroEL (see Figure 5).

Global averages of side-views indeed showed that the overall appearance of the GroEL cylinder changes only slightly upon binding of substrate protein (Figure 9B). An eigenvector–eigenvalue analysis of the data set revealed that the inter-image variability of the side-on views was more pronounced in the presence of substrate protein than in its absence (not shown). However, in none of these structural sub-classes were additional stain-excluding masses detected at the outer circumference of the cylinder. We conclude that the central density seen in end-views is related to bound rhodanese and is apparently enclosed within the cavity of the GroEL complex. The relatively small mass would be expected to be obscured in side-views since it is superimposed on the much larger mass of GroEL in projection. The location of the substrate-related density along the long axis of the GroEL cylinder remains to be determined.

We also analysed ternary complexes containing GroEL, GroES and substrate protein that had been prepared in the presence of Mg–ADP. In this case, sufficient particles in the side-on orientation could not be obtained. Averaging of end-views, however, again revealed a very similar stain-excluding mass in the central portion of the GroEL–GroES cylinder (Figure 9C). This was never observed when substrate protein was absent, although the conformation of GroEL was markedly changed upon GroES binding (see Figure 6). Interestingly, complexes formed with peroxisomal alcohol oxidase (Figure 9D) showed a density in the centre of GroEL that appeared somewhat larger than that seen with GroEL–rhodanese (Figure 9C). This would be expected considering the larger size of alcohol oxidase, 75 kDa, in comparison to rhodanese, 35 kDa.

Taken together, these results strongly suggest that folding substrate proteins bind within the central cavity of the GroEL cylinder. This would be consistent with models for the function of GroEL, which propose that a compact, intermediate form of the protein makes contact with several GroEL subunits (Hartl *et al.*, 1992).

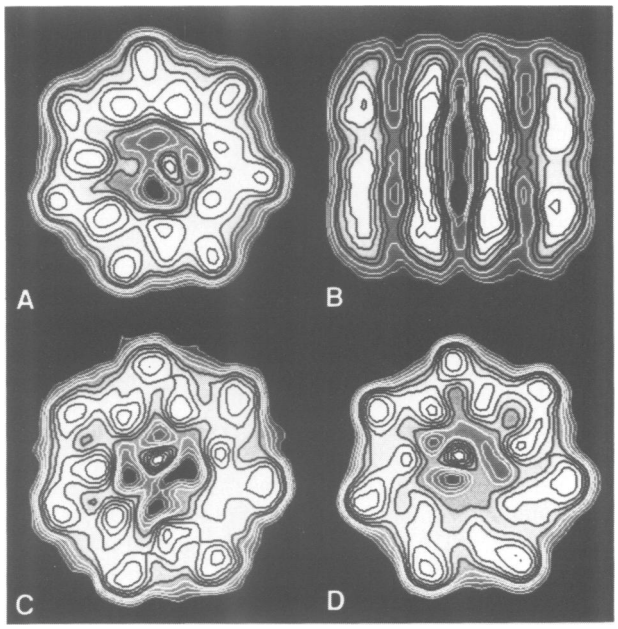

Fig. 9. Averages of electron microscopic images of GroEL and GroEL–GroES with bound substrate protein. (**A**), end-on view and (**B**), side-on view of GroEL–rhodanese; (**C**) end-on view of GroEL–GroES–rhodanese and (**D**), end-on view of GroEL–GroES–alcohol oxidase complexes formed as described in Material and methods. The total number of particles analysed was (A) 640, (B) 339, (C) 625 and (D) 687. The dimensions of the complexes are: A, sym. diameter 16.0 nm; B, 16.0 × 14.3 nm; c, sym. diameter 15.6; D, sym. diameter 15.0 nm. Averaging performed with multiple reference images gave essentially identical results.

Discussion

The function in protein folding of members of the GroEL/Hsp60 family critically depends on a co-chaperonin such as GroES or Hsp10. We have shown here that the GroEL and GroES oligomers form a 1:1 stoichiometric complex. GroES binding confers asymmetry to the GroEL cylinder and results in a large structural change of GroEL. GroES protects the C-terminal ~50 residues of half of the GroEL subunits from proteolytic cleavage. This segment may have a function in regulating the GroEL ATPase. Like free GroEL, the functional holo-chaperonin contains a central cavity that apparently accommodates the folding substrate protein.

In the absence of substrate protein, GroES is known to inhibit the ATPase activity of GroEL (Chandrasekhar *et al.*, 1986; Viitanen *et al.*, 1990; Martin *et al.*, 1991). While GroEL may release a bound polypeptide upon ATP-hydrolysis in the absence of GroES (Laminet *et al.*, 1990; Martin *et al.*, 1991; Viitanen *et al.*, 1991), this does not result in the productive folding of proteins such as rhodanese or citrate synthase, which have a strong tendency to aggregate (Martin *et al.*, 1991; Mendoza *et al.*, 1991; Zhi *et al.*, 1992). Our present results indicate that GroES fulfills its regulatory function by forming a 1:1 complex with the GroEL oligomer. GroES binds asymmetrically to one end-surface of the GroEL cylinder. This behaviour is not immediately expected since the GroEL double-ring is apparently symmetrical. Electron microscopic image analysis of the chaperonin of *Comamonas acidovorans* showed that the dumb-bell shaped GroEL subunits are arranged in the

double-ring such that the apparently smaller domains face each other (Zwickl *et al.*, 1990). Furthermore, the C-terminal region of GroEL can be proteolytically cleaved with equal efficiency from the subunits of both heptameric rings without compromising the oligomeric state of GroEL. Our binding experiments with GroEL-ΔC/2 indeed suggest that GroES is free to interact with either surface of the GroEL cylinder. It thus appears that GroES binding to one surface of GroEL induces a conformational change in the opposite surface, thereby strongly reducing the binding affinity for a second GroES oligomer. Consistent with this model, a pronounced structural alteration of GroEL was observed upon GroES binding.

It is thought that GroES couples the ATPase of GroEL with the function in protein folding (Viitanen *et al.*, 1990; Martin *et al.*, 1991). Binding of GroES is dependent on the presence of Mg−ATP or Mg−ADP. In the holo-chaperonin, either all or perhaps half of the GroEL subunits may be in the ADP-bound state. Upon binding a substrate protein, ADP−ATP exchange and subsequent ATP-hydrolysis would occur concomitantly with conformational changes induced in GroEL, allowing a polypeptide to be released in a manner productive for folding. Presumably, the high degree of conformational flexibility of GroEL, visualized in the complex with GroES, is of functional importance in this reaction. How exactly GroES exerts its allosteric effects on GroEL is unclear. The C-terminal region of GroEL may be involved in the mechanism by which GroES suppresses the uncoupled ATPase activity of substrate-free GroEL, but not in the GroES-dependent folding and release of the protein substrate. This sequence is at least partially surface-exposed and GroES binding protects it from proteolytic cleavage in half of the GroEL subunits, thereby allowing the formation of GroEL-ΔC/2. It is reasonable to assume that the C-terminal sequences are shielded in the GroEL heptamer, which is in direct contact with GroES. Although less probably, it is also possible that a conformational change induced by GroES results in protection of the opposite heptamer. The rate of ATP hydrolysis of GroEL-ΔC is nearly as slow as that of full-length GroEL, which has GroES bound. C-terminal truncation of the subunits of one heptamer is sufficient to cause this inhibition. GroES may therefore exert its inhibitory effect on the GroEL ATPase by interacting directly or indirectly with the C-terminal sequences of the subunits in one GroEL heptamer. Removal of these sequences may mimic this effect of GroES binding.

The C-terminal 16 amino acids of GroEL are entirely composed of methionine, alanine and glycine (Hemmingsen *et al.*, 1988), which is indicative of a high degree of flexibility. Similar motifs are found in the various mitochondrial Hsp60s, but not in the chloroplast rubisco subunit-binding protein (Hemmingsen *et al.*, 1988; Reading *et al.*, 1989). This segment plus at least 34 additional residues are deleted in GroEL-ΔC. The truncated GroEL has preserved its normal function in mediating protein folding *in vitro*, at least with rhodanese as the substrate protein, but it is conceivable that the functional interaction with other substrate proteins is impaired. This would be consistent with the observation that the C-terminal truncation of >27 residues of GroEL renders *E.coli* inviable (A.Horwich, personal communication). In contrast, removal of the 16 C-terminal residues alone does not result in a detectable loss of GroEL function *in vivo* (McLennan *et al.*, 1991).

The mechanism of GroEL-mediated protein folding is not yet understood in detail. Previous work has suggested that in the absence of ATP hydrolysis, the chaperonin stabilizes a conformational intermediate that may be relatively compact in comparison to the fully extended state (Martin *et al.*, 1991; Langer *et al.*, 1992; van der Vies *et al.*, 1992) and may contain secondary structure (Landry *et al.*, 1992). Since only 1−2 molecules of substrate protein associate per GroEL 14mer, it seems reasonable to assume that the folding protein binds to the central portion of the GroEL double-ring, making contact with several GroEL subunits. This is indeed suggested by the observation of an electron dense mass within the central space of the GroEL cylinder, which in all probability is related to the partially folded substrate protein. With the 75 kDa alcohol oxidase this central density appeared somewhat larger than with the 35 kDa protein rhodanese. Alcohol oxidase may exceed the size limit of a protein that can be readily accommodated within the GroEL double-ring. This could be one reason for the finding that this protein aggregates upon ATP-dependent release from GroEL (Evers *et al.*, 1992). Our data do not allow any conclusion as to the topology of the substrate protein relative to GroES. In principle, GroES and folding protein could associate at either the same or the opposite ends of the GroEL double-ring. It is also possible that the substrate protein, within the cavity of the chaperonin cylinder, can make contact with the central domains of either of the GroEL heptamers (Creighton, 1991). The observation that GroES binding is asymmetrical and causes a large conformational change of the opposite surface of the chaperonin cylinder suggests that both heptamers participate in GroEL function. In contrast, the Hsp60 of mammalian mitochondria is apparently active as a single 7mer ring, at least with ribulose bisphosphate carboxylase as substrate (Viitanen *et al.*, 1992). It has been proposed that partial folding may occur while the protein is sequestered from the bulk solution by GroEL (Creighton, 1991; Martin *et al.*, 1991). Regulation by GroES may result in the release of bound segments of the protein substrate in a coordinated ATP hydrolysis-dependent manner (Gray and Fersht, 1991; Bochkareva *et al.*, 1992). In this model, ATP-dependent release may be followed by rebinding of a segment of the polypeptide chain that may not yet have integrated properly into the folding structure. Release and folding may thus occur in a controlled, step-wise process.

Materials and methods

Protein purification

GroEL and GroES were purified from a GroE-overproducing strain of *E.coli* harbouring the plasmid pOF39 (Fayet *et al.*, 1986; Viitanen *et al.*, 1990). Protein concentrations were determined based on quantitative amino acid analysis and colorimetric protein determination (Bradford, 1976).

^{3}H-labelling

Purified GroES was radiolabelled by reductive methylation using [^{3}H]$NaBH_4$. GroES was dissolved in 0.2 M $NaBO_3$ pH 8.9 at a concentration of 71.4 μM and formaldehyde was added to a final concentration of 10.3 mM, corresponding to a 2-fold excess over lysine residues. After addition of 2.5 mM [^{3}H]$NaBH_4$ (11 Ci/mmol; NEN), the sample was incubated for 15 min at 4°C. The reaction was stopped by addition of 20 mM $(NH_4)_2SO_4$. Labelled GroES was separated on a PD10-column (Pharmacia) equilibrated with 10 mM MOPS−KOH pH 7.2 and 50 mM KCl (buffer A). GroES-containing fractions were pooled. The specific activity of the re-purified protein was 95 000 c.p.m./pmol oligomer.

Native PAGE

GroEL−GroES complex formation was analyzed by native PAGE using a 3−10% acrylamide gradient in 80 mM MOPS−KOH pH 7.2, 1 mM

Mg-acetate and 2 mM ATP or ADP. Electrophoresis was carried out for 12 h at 4°C at 20 mA and a constant voltage of 150 V. The running buffer contained 80 mM MOPS–KOH pH 7.2, 1 mM Mg-acetate and 0.1 mM ATP or ADP.

Rhodanese refolding

Rhodanese from bovine liver (~95% pure; Sigma) was denatured in 6 M guanidinium chloride as described by Langer *et al.*, (1992) and diluted 70- to 150-fold into buffer A to a final concentration of 0.46–1.4 μM containing 0.49–0.89 μM GroEL or GroEL–GroES complex, as specified in the figure legends. Enzyme activities were determined according to Martin *et al.*, (1991) and Tandon and Horowitz (1989).

Electron microscopy

Unless otherwise indicated, GroEL–GroES complexes for electron microscopic analysis were prepared by incubating GroEL and GroES (0.89 μM and 9 μM with respect to oligomers) at 25°C in buffer A containing 5 mM Mg acetate and 1 mM ADP. For the formation of ternary complexes of GroEL–GroES–substrate protein rhodanese and alcohol-oxidase (from *Hansenula polymorpha*; ~95% pure) were denatured as described previously by Langer *et al.* (1992) and Evers *et al.* (1992) and diluted 100-fold or 60-fold into GroEL containing solution to a final concentration of 2.7 μM or 1.8 μM (monomer), respectively. Then Mg–ADP and GroES were added as above. Control reactions in the absence of substrate protein were routinely prepared in the presence of 60 mM guanidinium chloride. GroEL complexes were isolated by gel chromatography on Sephacryl S300 columns (0.5 × 6 cm) equilibrated with buffer A. Fractions of 110 μl were collected and analysed by SDS–PAGE.

GroEL and the various complexes of GroEL with GroES and substrate protein were negatively stained with 1% uranyl acetate. Electron micrographs were recorded with a Philips EM 420 and CM 12 at nominal magnifications between 35 000 and 49 000×. Since care was taken not to preirradiate the specimen areas to be imaged, the total dose was kept below 4000 e^-/nm^2. Suitably covered areas of the micrographs were scanned by densitometry using an EIKONIX CCD camera with a step size of 15 μm. Individual particles were selected interactively from digitized micrographs using the Metheus Omega 445 raster-graphics display system. From each of the different preparations analysed between 50 and 900 molecular images were selected. The images were aligned with respect to translation and orientation using standard correlation techniques; the alignment was refined iteratively. Averaging performed with multiple reference images gave essentially identical results. In order to detect inter-image structural variations the aligned images were subjected to a classification procedure based on eigenvector-eignevalue data analysis (van Heel and Frank, 1981). All computations were performed using either the SEMPER (Saxton *et al.*, 1979) or EM (Hegerl and Altbauer, 1982) software systems.

Miscellaneous

The following procedures were carried out according to published methods: determination of GroEL ATPase-activities (Lanzetta *et al.*, 1978; Lill *et al.*, 1990); trichloroacetic acid (TCA) precipitation of proteins (Bensadoun and Weinstein, 1976); SDS–PAGE (Laemmli, 1970); electrotransfer to nitrocellulose (Towbin *et al.*, 1979); immunolabelling using the luminescence based detection system ECL (Amersham) (Vachereau, 1989).

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