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Multivalent Binding of Nonnative Substrate Proteins by the Chaperonin GroEL

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Summary

The chaperonin GroEL binds nonnative substrate protein in the central cavity of an open ring through exposed hydrophobic residues at the inside aspect of the apical domains and then mediates productive folding upon binding ATP and the cochaperonin GroES. Whether nonnative proteins bind to more than one of the seven apical domains of a GroEL ring is unknown. We have addressed this using rings with various combinations of wild-type and binding-defective mutant apical domains, enabled by their production as single polypeptides. A wild-type extent of binary complex formation with two stringent substrate proteins, malate dehydrogenase or Rubisco, required a minimum of three consecutive binding-proficient apical domains. Rhodanese, a less-stringent substrate, required only two wild-type domains and was insensitive to their arrangement. As a physical correlate, multivalent binding of Rubisco was directly observed in an oxidative cross-linking experiment.

Introduction

The GroEL-GroES chaperonin system, employing steps of ATP binding and hydrolysis, facilitates folding of a variety of proteins by binding nonnative conformations in the hydrophobic cavity of an open GroEL ring and then triggering productive folding upon subsequent binding of GroES to the same ring as polypeptide (for reviews, Horovitz, 1998; Sigler et al., 1998). The requirements and constraints of the polypeptide binding step are not well delineated. Recent studies of the dynamically cycling chaperonin system indicate that polypeptide normally binds to the open *trans* ring of an asymmetric GroEL-GroES-ADP complex (Sparrer and Buchner, 1997; Rye et al., 1999). This complex has been analyzed crystallographically, revealing that the terminal hydrophobic apical domains of the seven GroEL subunits of the *trans* ring are fully accessible to nonnative polypeptide (Xu et al., 1997). Moreover, this ring is isomorphous to the rings of unliganded GroEL (Braig et al., 1994), indicating that investigations of polypeptide binding by unliganded GroEL are likely to be relevant to binding in the presence of the cycling system.

Studies of polypeptide binding to both GroEL-GroES-ADP complexes and unliganded GroEL indicate that nonnative substrate proteins, for example Rubisco or malate dehydrogenase (MDH), bind in a rapid bimolecular manner ($k \sim 1–2 \times 10^7\ M^{-1}sec^{-1}$) (Fenton and Horwich, 1997; Rye et al., 1999). While substrates become stably bound ($K_d \sim 1–10$ nM) (e.g., Goloubinoff et al., 1989; Ranson et al., 1997), they occupy unstable conformations, as revealed both by susceptibility to proteolysis and by hydrogen-deuterium exchange experiments showing amide proton protection factors generally ranging from 2–50 (Fenton and Horwich, 1997). Other aspects of polypeptide binding are less well understood, particularly the role of multivalency in both initial binding and subsequent folding. Nonnative proteins likely present an ensemble of conformations to GroEL. The chaperonin might take advantage of its multiple apical domains to bind a collective of these species. Alternatively, the multivalent binding surface could select specific, perhaps less folded, conformations that interact with multiple domains, and, hence, with higher affinity; in the setting of rapid interconversion of unfolded species, this would shift the population toward more efficiently bound states (Walter et al., 1996). Partial unfolding could even be directly supported by multivalency if initially bound conformations were "stretched" or "annealed" to produce more stable, multiply interacting species (Todd et al., 1996; Zahn et al., 1996). Indeed, the last possibility may also be pertinent to the folding phase of the cycle, as a recent study observed deprotection of a small number of protected amide protons in GroEL-bound Rubisco upon addition of ATP-GroES, suggesting that GroES binding to the polypeptide-bound ring, attended by large-scale rigid-body movements of the apical domains, could stretch and further unfold a nonnative polypeptide if it were bound to multiple apical sites (Shtilerman et al., 1999). Here, we have addressed the question of whether the substrates, Rubisco, MDH, and rhodanese, are bound multivalently by GroEL rings, using functional studies with mutant GroEL complexes and physical studies employing cysteine cross-linking.

Results

Production of Covalent GroEL Rings

Assessment of the contribution of individual subunits and multivalency to polypeptide binding and productive folding by GroEL would be possible if GroEL rings were generated that contained specific numbers and arrangements of wild-type and binding-mutant apical domains.

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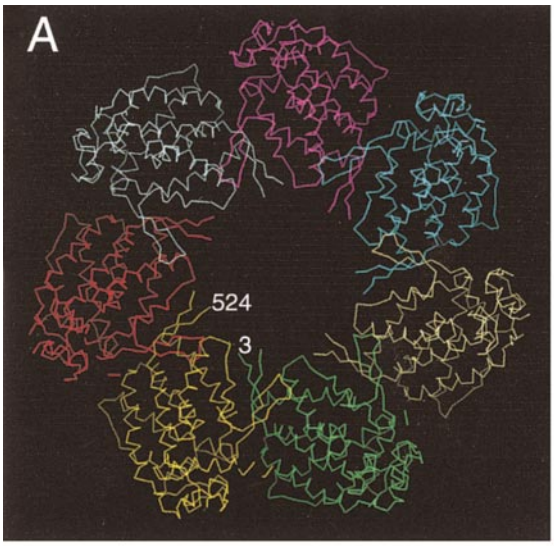
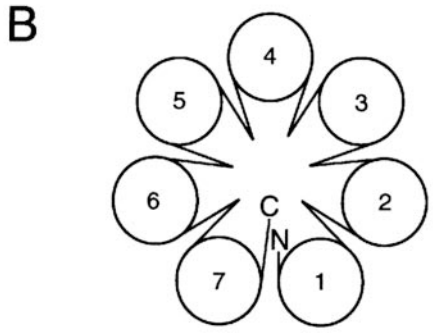
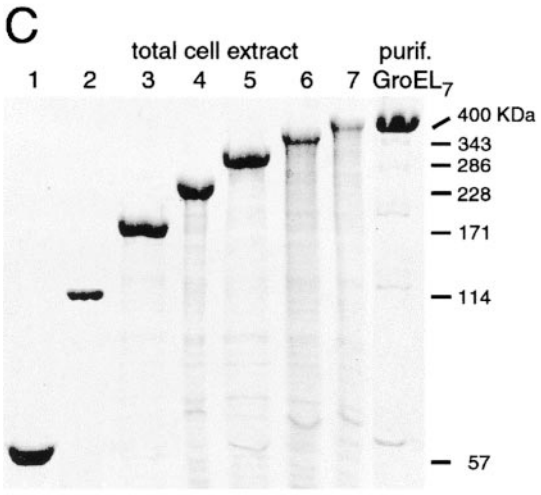






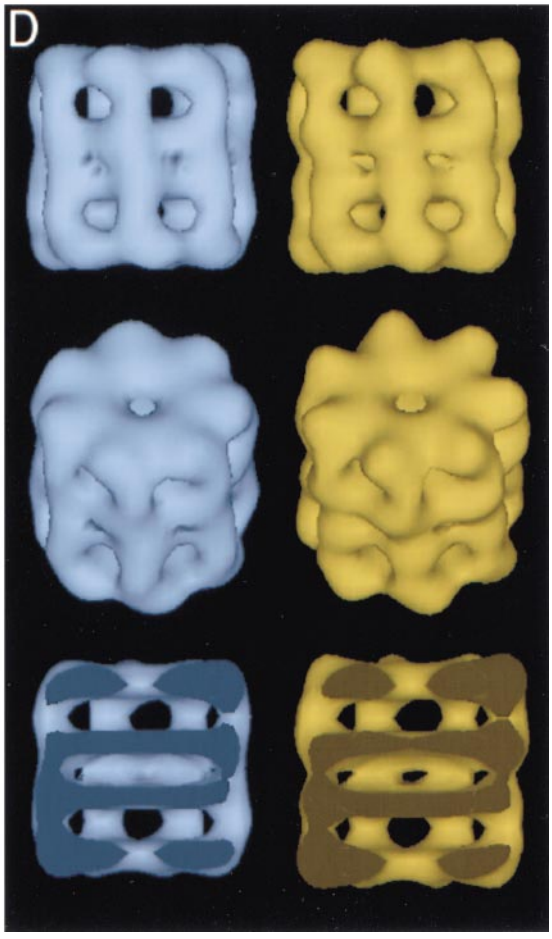


Figure 1. Production of GroEL Rings as Single Polypeptides—Construction and Analysis

(A) Structural basis of strategy for tandem joining of GroEL coding sequences. Cα trace of an unliganded GroEL ring at the level of its equatorial domains, showing the crystallographically resolvable termini of the subunits. An additional 24 COOH-terminal residues lying beyond 524 are not crystallographically resolvable. The apparently flexible tail of one subunit might thus be joined through residue 548 to the NH_2 terminus (residue 2) of the neighboring subunit without affecting structure or function (see text). (Model produced in O; Jones et al., 1991.)

(B) Schematic diagram of GroEL ring produced from tandemly arranged GroEL coding sequences as a single polypeptide chain, illustrating the connections between what would normally be the COOH terminus of one subunit and the NH_2 terminus of its counterclockwise-situated neighbor.

(C) SDS-PAGE analysis of lysates of bacteria expressing intermediate constructs and the final seven unit construct. Lanes 1–7, strains bearing *trc* plasmids encoding the indicated number of tandemly arranged GroEL subunits, grown at 23°C without induction, were directly solubilized and fractionated on a 6% SDS-PAGE gel. Right-hand lane, covalent GroEL complex purified under native conditions from the strain harboring the seven unit construct (lane 7).

One means of achieving such combinations would be to produce a GroEL ring as a single polypeptide chain containing seven covalently linked subunits. The feasibility of generating covalent rings was supported by inspection of the crystallographic model of an unliganded GroEL ring (Figure 1A). Residues 524 and 3 of adjacent subunits (the last and first resolved residues) lie ~15 Å from each other at the wall of the central cavity in the equatorial domain. In addition, there are 24 unresolved COOH-terminal residues, which extend into the central cavity and have been collectively recognized in cryo-EM studies as a mass at the level of the equatorial domains (e.g., Rye et al., 1999). This flexible terminus might thus be accessible to the NH_2 terminus (residue Ala2) of the neighboring subunit in the ring, suggesting that their covalent connection (as diagrammed in Figure 1B) might be possible without major disturbance of architecture or function.

Units of coding sequence of GroEL were added one by one to the GroEL coding region in a plasmid that cooverproduces GroES and GroEL from a *trc* promoter (Fenton et al., 1994), to produce a continuous GroEL open reading frame containing seven consecutive GroEL units, encoding a 400 kDa protein. Units were joined through alanine and glycine-containing segments of two to four residues, with each such joint carrying a unique restriction site enabling replacement of wild-type units with mutant versions. During construction, plasmids encoding increasing numbers of units were monitored for expression of GroEL fusion proteins of the appropriate size in *recA*$^-$ DH5α (Figure 1C, lanes 1–7). The complete seven unit sequence was then expressed at 25°C in the absence of inducer. The predicted 400 kDa species was found in the soluble fraction of cells broken under native conditions. In anion exchange chromatography, a substantial amount of this protein eluted in the flow-through fraction and was observed, upon gel filtration, to migrate with a molecular size of several million daltons (data not shown). This material likely represents misfolded and/or misassembled molecules that form low-order aggregates. The remaining amount of the species, however, eluted from anion exchange exactly as wild-type GroEL, and, upon gel filtration, migrated as an 800 kDa protein, identically to wild-type GroEL. This suggested that the 400 kDa species had assembled into the double-ring complex characteristic of GroEL. This was confirmed by cryo-EM and three-dimensional reconstruction of the purified protein, showing a complex of two back-to-back seven-membered rings that was indistinguishable from wild-type GroEL at ~30 Å resolution (Figure 1D). Thus, constituent subunits of these rings, though physically joined, have apparently undergone correct folding and oligomeric assembly.

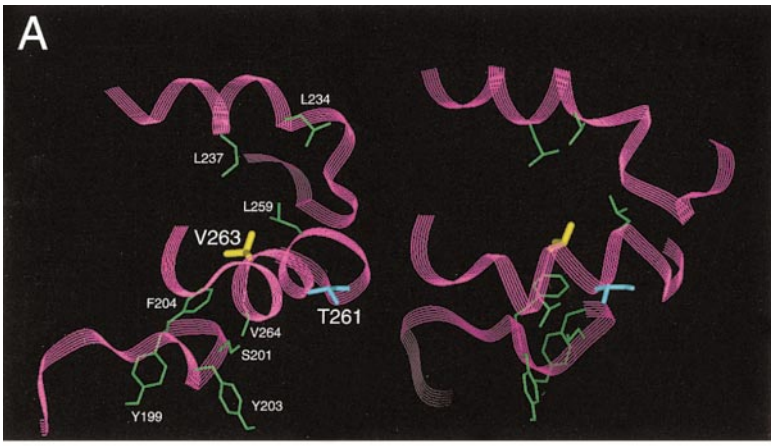
Consistent with ostensibly normal structure and function of these double ring assemblies, transformation of the seven unit encoding plasmid into a GroEL-deficient *Escherichia coli* strain, LG6 (Horwich et al., 1993), in which the *groE* operon promoter in the bacterial chromosome is replaced by a *lac* promoter, rescued growth in the absence of IPTG. Such rescue occurred under the same growth conditions, 25°C and absence of induction, in which the covalent GroEL had been isolated, and produced approximately the same number of colonies as transformation with a plasmid encoding noncovalent wild-type GroEL. Direct solubilization of individual LG6 colonies transformed with the seven unit construct revealed presence of the 400 kDa covalent species, without any noncovalent monomeric GroEL subunits detectable by Coomassie staining (data not shown), indicating that rescuing function was supplied by the covalent GroEL and not by proteolytically cleaved monomer-containing molecules. Consistent with this, plasmids encoding four, five, and six GroEL units were unable to rescue the LG6 strain.

Mutant Covalent GroEL Molecules Tested In Vivo

Rescue of GroEL-deficient cells was next tested with a series of seven unit constructs bearing various numbers and arrangements of subunits containing the apical domain substitution, V263S. V263 lies in the center of the peptide binding surface of the apical domain on an α helix and faces upward toward an overlying α helix that forms the inlet to the central cavity when the GroEL ring is in an open "acceptor" state (Figure 2A). Earlier studies observed that the V263S substitution was lethal and that, when present in all subunits of a mutant tetradecamer, it abolished both polypeptide binding and association in the presence of ADP of the cochaperonin GroES (Fenton et al., 1994). Nevertheless, a 30 Å cryo-EM map of the V263S tetradecamer (data not shown) was indistinguishable from that of wild-type GroEL.

Seven unit constructs were produced carrying from one to five V263S-substituted units and transformed into GroEL-deficient LG6 cells. A construct containing a single mutant unit (U1 263) rescued as well as the wild-type seven unit construct, with several thousand colonies obtained per dish (corresponding to ~25 ng transforming DNA; Figure 2B). Likewise, the substitution of two or three consecutive mutant units rescued as well as wild-type (e.g., U1,2 263 or U1,2,3 263; Figure 2B). Such alterations were placed at a different position in the ring and achieved the same rescue (U4,5 263 and U4,5,6 263). This supports, in functional terms, the cryo-EM observation that the covalent ring has 7-fold symmetry despite the presence of six covalent connections (and absence of a seventh, between units seven and one). (It should be noted that the rotational orientation of the rings relative to each other is unknown, although, given the symmetric behavior within a ring, it seems likely that this is random.) Finally, in contrast with efficient rescue by four or more consecutive wild-type units, rescue by a construct with three consecutive wild-type units was diminished to ~5%, totaling only 50–100 colonies per dish (see, however, section on binding in vitro).

(D) Cryoelectron microscopic image reconstructions comparing wild-type GroEL (left) and covalent GroEL complex (right), shown from the side (top panels), tipped forward (middle panels), and cut open (bottom panels) to reveal the internal cavities. The structures are very similar. There may be a slight difference in the positions of the apical domains between the covalent complex and the wild-type, but it is not significant at this resolution.



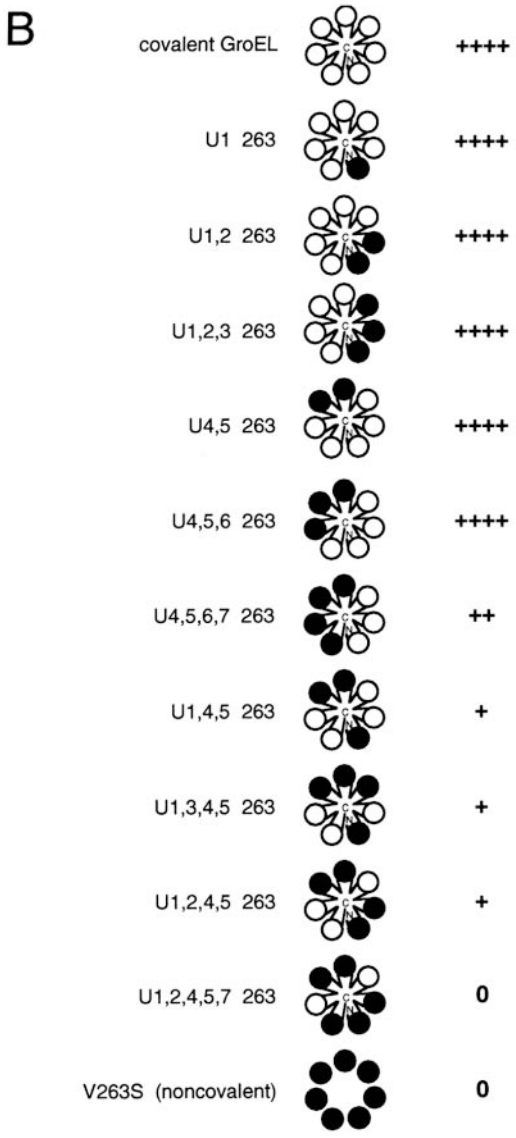


Figure 2. Function In Vivo of GroELs with Covalent Rings Bearing Various Numbers and Arrangements of Wild-Type and Mutant V263S Subunits

(A) Position of V263 residue, whose substitution in all subunits of a tetradecamer abolishes polypeptide binding. Model from unliganded GroEL of the polypeptide binding surface of the apical domains of two adjacent GroEL subunits, viewed from the central cavity, showing V263 (yellow) and other residues whose mutation in all 14 subunits abolishes polypeptide binding (green). Cyan side chain of T261 indicates residue where a cysteine was substituted in the experiment described in Figure 8. (Model produced in InsightII, BioSym.)

(B) Results of in vivo rescue experiments with various GroEL covalent constructs. Constructs with various arrangements of wild-type units (open circles) and V263S-substituted units (filled circles) were transformed into the strain LG6, which bears a *lac*-driven chromosomal *groE* operon and becomes GroEL deficient and growth arrested in the absence of IPTG. The construct name designates the position of the units bearing the 263 mutation. The number of colonies obtained in the absence of IPTG, relative to transformation with the same amount of plasmid encoding noncovalent wild-type GroEL, is designated by the number of (+) marks, with 4+ indicating the number of colonies produced by the plasmid encoding noncovalent wild-type GroEL.

Whether the V263S mutant apical domains in these constructs are completely nonfunctional with respect to action on the critical substrate proteins in vivo or whether they exhibit residual activity was unclear. To address this issue, we asked whether introduction of a second apical alteration, L237E, into subunits already containing V263S would have an additive effect to that of V263S alone. The L237E substitution lies in the α helix

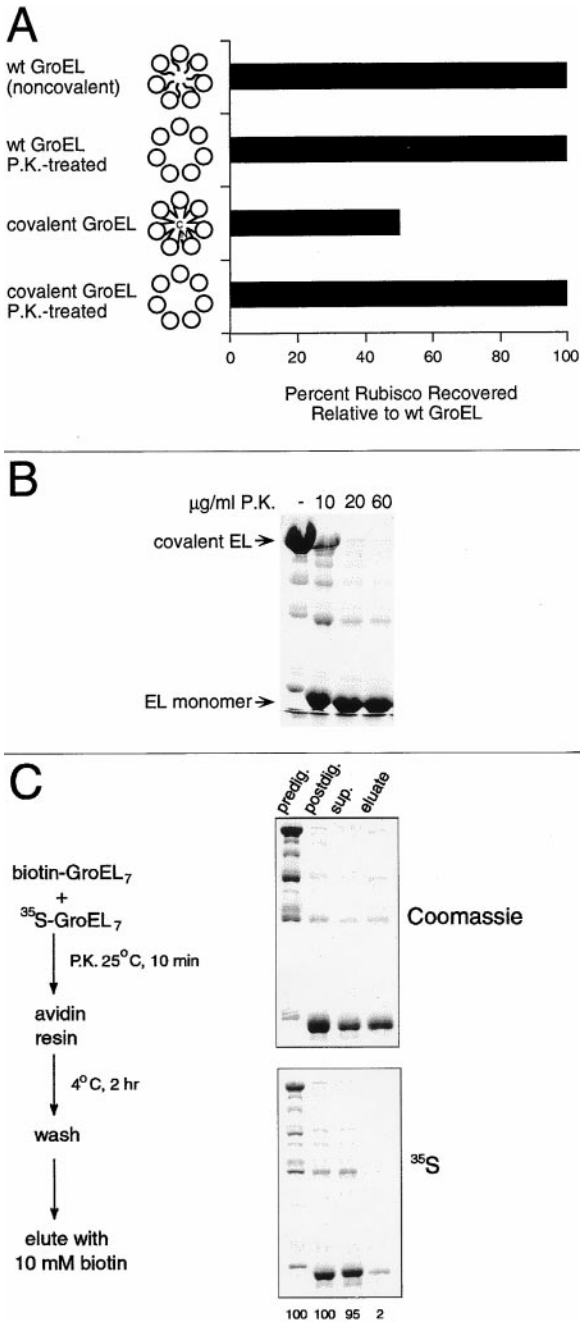


Figure 3. Polypeptide Binding In Vitro Is Reduced by the Covalent Connections, but Proteolytic Removal Restores Binding to Normal without Destabilizing the Ring

(A) Extent of Rubisco binding to untreated and proteinase K–treated noncovalent and covalent GroEL complexes. ^{35}S-labeled Rubisco was unfolded in acid and diluted into buffer containing the indicated GroEL complexes. The mixtures were then fractionated by gel filtration and the amount of Rubisco recovered in the GroEL-containing fractions quantitated by liquid scintillation counting. Typically, 50%–60% of input radioactivity was recovered with wild-type noncovalent GroEL.

(B) SDS-PAGE analysis of proteinase K-treated covalent GroEL complex, showing conversion of 400 kDa GroEL to ~55 kDa species.

(C) Partial proteolysis does not destabilize GroEL rings or allow exchange of GroEL subunits. As diagrammed, biotinylated and ^{35}S-labeled covalent complexes were mixed and subjected to partial proteinase K digestion as in (B) (lanes 1 and 2, before and after protease). The mixture was then incubated with avidin beads, after which the initial supernatant (lane 3) and the biotin eluate (lane 4) of the beads were analyzed in 6% SDS-PAGE with Coomassie staining (top panel) and quantitated by Phosphorimager analysis (bottom panel). Nearly all of the radioactive subunits were recovered in the supernatant, indicating that they had not exchanged with the biotinylated complex.

at the inlet to the central cavity (Figure 2A) and produces, on its own in a noncovalent construct, the same effects as V263S. This second substitution was added to each of the mutant units in U4,5, U4,5,6, and U4,5,6,7 263 constructs, and the single (263) and double (237 + 263) mutant constructs were then tested side by side for rescue. Identical results were obtained. In particular, the efficient rescue by U4,5 and U4,5,6 was not reduced by double substitution, and the reduced rescue of U4,5,6,7 was not further diminished by presence of the additional L237E substitutions. This supports the conclusion that the single V263S alteration is sufficient to functionally inactivate an individual apical domain.

We next examined the effect of nonconsecutively placed wild-type units. When four wild-type units were arranged as two separated pairs (U1,4,5 263), the number of rescued clones dropped to near zero (10–20 per dish; Figure 2B). Likewise, when three wild-type subunits were arranged such that a pair of wild-type units was separated from a third unit, the low level of rescue observed with three consecutive units was further diminished to near zero. Thus, in an in vivo setting, wild-type units in consecutive arrangements were favored over nonconsecutive ones. As shown below by experiments in vitro, these effects appear likely to reside at the level of polypeptide binding as opposed to GroES association, presumably reflecting the binding requirements of the most GroEL-dependent protein(s) that mediate essential functions in the cell.

Polypeptide Binding by Purified GroEL Molecules

Presence of Covalent Connections Reduces the Extent of Polypeptide Binding, but Excision of the Connections by Partial Proteolysis Restores Binding to Normal

While the seven unit wild-type plasmid could efficiently rescue growth of GroEL-deficient cells (Figure 2B), we observed that the purified wild-type covalent GroEL complex was not as efficient as natural noncovalent GroEL in binding radiolabeled Rubisco, exhibiting an extent of binding only ~50% that of noncovalent GroEL, as measured by gel filtration (Figure 3A). Considering that the only difference in composition between the covalent assembly and noncovalent GroEL is the connections between subunits, we surmised that either the connections produced a steric constraint, e.g., the connected tails might project up into the apical portion of the central cavity, blocking access of the polypeptide to the apical binding surface, or the connected tails might prevent an as yet unrecognized allosteric adjustment that occurs upon initial substrate interaction and is required for stable association of polypeptide. While we could not resolve which of these effects might be involved, we elected to selectively excise the covalently

connected tails using partial proteolytic digestion with proteinase K, as originally described for wild-type GroEL by Langer and coworkers (Langer et al., 1992). Such treatment, typically removing ~20 COOH-terminal residues from wild-type subunits, has no effect on GroEL function in polypeptide binding or GroES/ATP-directed folding in vitro (Langer et al., 1992; Figure 3A). Correspondingly, genetic deletion of the tail coding sequence (27 codons) does not interfere with function in vivo (Burnett et al., 1994). When covalent GroEL was subjected to partial proteolysis, the complex remained intact and indistinguishable from wild-type as observed by gel filtration and electron microscopy (data not shown) and, in SDS-PAGE analysis, as expected, monomer-sized subunits were observed (Figure 3B). Using higher percentage gels, these monomers were observed to migrate faster than wild-type GroEL subunits, comigrating with subunits of wild-type noncovalent GroEL that had been similarly treated with proteinase K (data not shown). Thus, the tails are not only incised but are proteolytically removed from the covalent assembly by the proteinase K treatment. After purification of the "clipped" GroEL by gel filtration, it was tested for ability to bind nonnative Rubisco. The extent of binding was now observed to be restored to normal (Figure 3A), indicating that removal of the covalent connections allows normal polypeptide binding.

It seemed critical to establish that excision of the covalent connections was not associated with destabilization of the rings or exchange of subunits, which could potentially allow rearrangement of their genetically programmed order. To test for exchange, we coincubated two versions of covalent wild-type GroEL during partial proteolysis, one metabolically labeled with ^{35}S-methionine and the other lightly labeled with NHS-biotin (Figure 3C). Following the proteolysis step, the mixture was incubated with avidin beads to capture GroEL complexes bearing biotinylated subunits. The beads were recovered, washed, and eluted with biotin to determine whether ^{35}S-methionine-bearing subunits had exchanged into biotin-bearing complexes during proteolysis. Two percent of the input counts were detected in the biotin eluate (Figure 3C). Thus, the radiolabeled subunits were nearly quantitatively recovered in the supernatant from the original incubation mixture. This indicates that proteolytic removal of the covalent connections does not destabilize the GroEL ring and lead to exchange of subunits. Such a conclusion is also supported by observation (below) that different arrangements of the same number of wild-type and mutant subunits exhibited distinct binding.

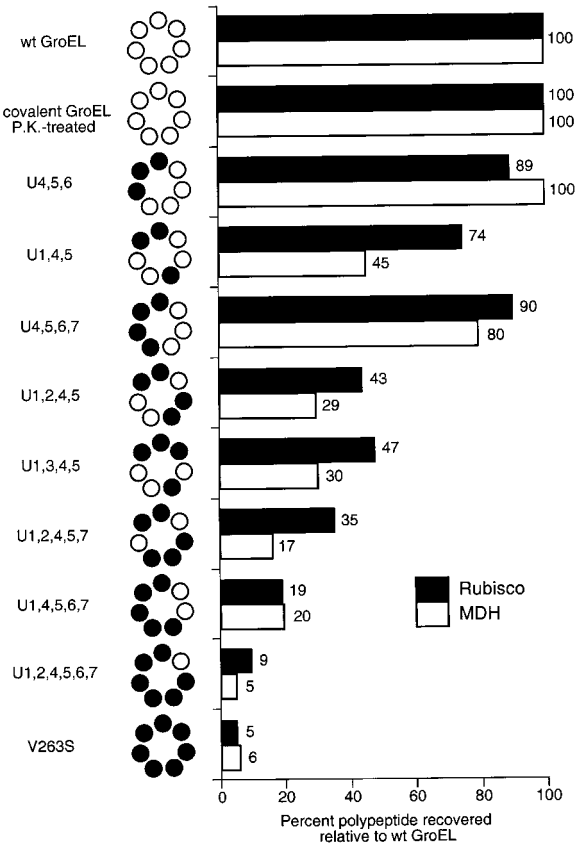


Figure 4. Binding of Nonnative Rubisco and MDH to Purified Protease-Treated Mutant GroELs

^{35}S-labeled Rubisco, unfolded in acid, or MDH, unfolded in guanidine-HCl, were diluted into buffer containing the indicated mutant complexes (compositions designated as in Figure 2B). Binary complexes with GroEL were isolated by gel filtration, and the amount of bound substrate was quantitated as described in the Experimental Procedures. The recovered amounts of Rubisco and MDH were normalized to those bound by noncovalent wild-type GroEL. The percentages bound were reproducible to within a few percent.

Polypeptide Binding by Purified 263 Mutant Complexes

MDH and Rubisco Require At Least Three Consecutive Binding-Proficient Subunits

The various purified 263 mutant-containing complexes, with their covalent connections proteolytically removed, were tested for ability to bind the subunits of two stringent (i.e., GroEL-GroES-ATP-dependent) substrate proteins, pig heart mitochondrial malate dehydrogenase (MDH; 33 kDa subunit) and Rubisco from *Rhodospirillum rubrum* (52 kDa subunit). The native forms of the substrate proteins, both homodimers, were unfolded in guanidine-HCl or acid, respectively, and then diluted into neutral pH buffer containing the respective mutant GroEL complexes. After incubation (20 min at 25°C), the mixtures were gel filtered, and the amount of input protein comigrating with the mutant GroEL complex was determined relative to wild-type. The results of these tests with respect to arrangement of wild-type subunits were similar to those in vivo, with consecutive binding-proficient subunits favored over nonconsecutive. In particular, nonconsecutive arrangements of three or four wild-type subunits were associated with substantial reduction in the amount of binding of either MDH or Rubisco relative to consecutive arrangement of the same number of subunits (Figure 4). However, in contrast with only weak rescue in vivo, a nearly full extent of binding for both MDH and Rubisco was observed with a complex with only three consecutive wild-type subunits. This is almost certainly the result of improved binding by the proteolytically treated complexes as compared with complexes studied in vivo, which retained the covalent connections. Directly supporting this, several of the complexes exhibited a much reduced extent of binding when tested in vitro with their covalent connections left

intact. For example, whereas the proteolytically clipped version of a complex with three consecutive wild-type subunits exhibited 90% binding, the covalent version exhibited only ~20% binding. We conclude that, as with complexes containing only wild-type subunits (Figure 3A), mutant complexes retaining the covalent connections suffer a significant loss of binding function relative to complexes from which such connections are removed. Thus, the in vivo study is likely to overestimate the minimum requirement (four) of wild-type subunits needed for full rescue. By contrast, when the connections were removed, resembling the physiologic situation, the minimum number of consecutive wild-type subunits sufficient for full extent of Rubisco and MDH binding is three.

Rhodanese Requires At Least Two Binding-Proficient Subunits

The binding behavior of rhodanese, a monomeric mitochondrial sulfurtransferase (33 kDa), was also examined. Refolding of this protein is less stringent than that of MDH and Rubisco, dependent on GroEL and GroES but supported by either ATP or ADP (albeit that refolding in ADP is much slower than in ATP; see, e.g., Weissman et al., 1996). Here, only two wild-type subunits were needed for full binding (Figure 5), with one proficient subunit exhibiting much reduced binding. In further contrast to MDH and Rubisco, efficient rhodanese binding did not require a contiguous arrangement of binding-proficient subunits. For example, while there was 64% recovery of ^{35}S-rhodanese with a complex containing two contiguous wild-type subunits (U1,4,5,6,7), 73% recovery was observed when two wild-type subunits were placed opposite each other in the ring (U1,2,4,5,7) (Figure 5). This ability of separated wild-type subunits to support binding may be consistent with an earlier proposal (Hlodan et al., 1995) that the two similarly folded domains of native rhodanese become bound in the nonnative state to two separate surfaces of the GroEL ring, to prevent domain swapping of the homologous secondary structures. If this is the case, it apparently can be accomplished by either adjacent or separated binding-proficient subunits.

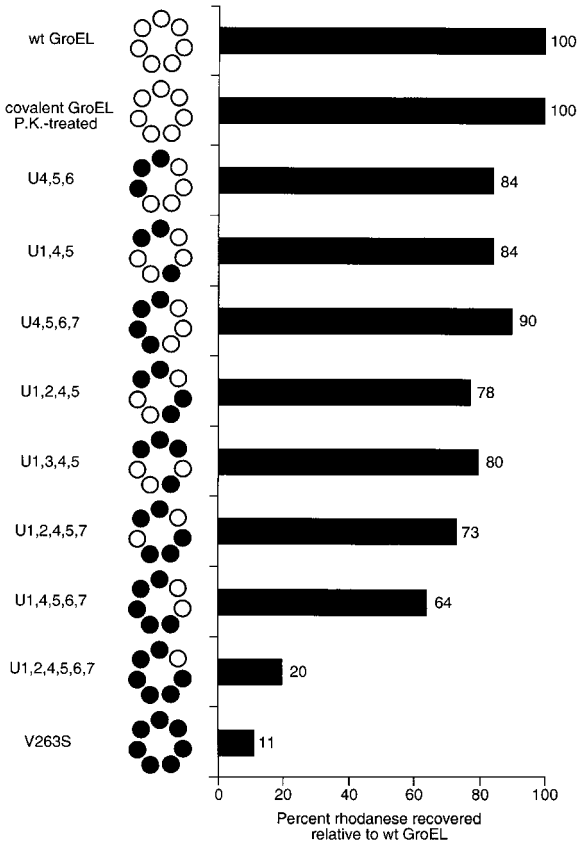


Figure 5. Binding of Nonnative Rhodanese to Purified Protease-Treated Mutant GroELs

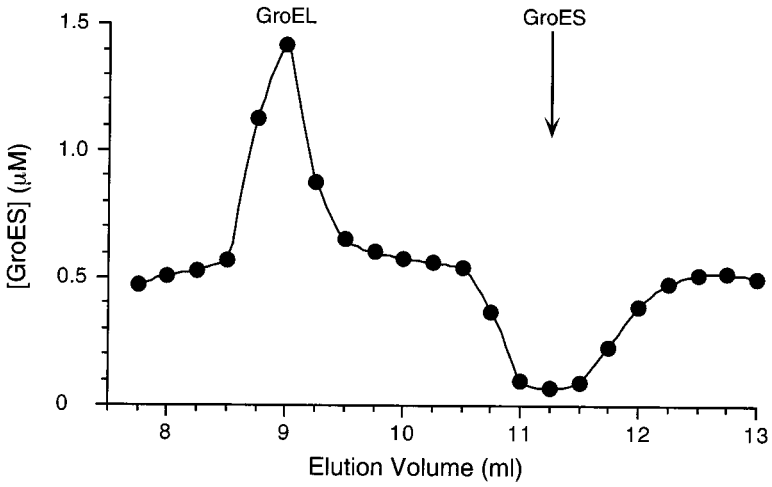
^{35}S-labeled rhodanese, unfolded in guanidine-HCl, was diluted into buffer containing the indicated mutant complexes (compositions designated as in Figure 2B). Binary complexes with GroEL were isolated by gel filtration, and the amount of bound substrate was quantitated by measuring ^{35}S radioactivity. The recovered amounts of rhodanese were normalized to those bound by noncovalent wild-type GroEL.

GroES Binding by 263 Mutant Complexes in ATP Is Normal As Long As There Is More Than One Wild-Type Subunit

The various proteolytically clipped 263 mutant-containing complexes were also tested for GroES binding in the presence of ATP, the physiologically relevant nucleotide for driving the GroEL-GroES reaction cycle, using Hummel-Dreyer analyses (Figure 6). These tests provide a relative measure of the affinity of GroEL for GroES under essentially steady-state conditions in a gel filtration column, in which GroES can bind freely to migrating GroEL in the presence of ATP and then release from it following *cis* ring ATP hydrolysis and *trans* ring ATP binding (Rye et al., 1997). The extent to which various complexes can bind GroES can thus be estimated by the amount of radioactive GroES coeluting with GroEL from the column. A G4000 gel filtration column was equilibrated in 0.5 μM ^{35}S-labeled GroES and 2 mM ATP, a GroEL complex was applied, and radioactivity eluting with GroEL, as well as the corresponding deficiency of radioactivity eluting with GroES, was measured (see Figure 6A for typical elution profile). As shown in Figure 6B, when the amounts of radioactivity eluting with the various complexes were compared, no effect of the presence of 263 mutant subunits was observed, unless either six or seven mutant subunits were present, with the six mutant subunit complex binding ~60%, reflecting diminished affinity, and the fully mutant complex binding ~20% as much GroES as wild-type. Thus, it seems that two or more wild-type apical domains are sufficient to produce normal GroES binding under these conditions and even one wild-type apical domain confers a significant degree of affinity for GroES. Whether the remaining one or two wild-type subunits are triggering cooperative binding by their mutant neighbors is not known.

MDH Refolding by Mutant Complexes Directly Parallels Polypeptide Binding Competence

The activity of the 263 mutant complexes in protein folding was examined, employing a complete chaperonin reaction mixture (i.e., GroEL, GroES, and ATP) to



GroEL complex	mole GroES bound / mole GroEL
wt GroEL	1.05
covalent GroEL P.K.-treated	0.96
U4,5,6	0.95
U1,4,5	0.86
U4,5,6,7	0.88
U1,2,4,5	0.88
U1,3,4,5	0.91
U1,2,4,5,7	0.97
U1,4,5,6,7	0.87
U1,2,4,5,6,7	0.61
V263S	0.18

Figure 6. Affinity of Mutant GroEL Complexes for GroES Measured by Hummel-Dreyer Analysis

Relative affinity of GroEL complexes for GroES in the presence of ATP was measured by a Hummel-Dreyer analysis, in which the indicated complex was applied to a G4000 SW_{xl} gel filtration column equilibrated in 0.5 μM ^{35}S-labeled GroES and 2 mM MgATP. Top panel shows elution profile of radioactivity when wild-type GroEL was applied to such a column, with a peak at the elution position of GroEL, corresponding to GroES associated with the chaperonin, and a trough at the position of GroES, corresponding to the amount of GroES associated with the GroEL peak. Botton panel shows the amount of GroES bound to the various complexes relative to wild-type. The various mutants exhibited essentially the same GroES binding as wild-type, except for U1,2,4,5,6,7, and V263S, which showed reduced binding.

measure refolding of MDH diluted from denaturant (Figure 7). Because the previous experiments indicated that GroES binding was unaffected in many of the mutant complexes in which polypeptide binding was reduced, we predicted that, for such mutants, the effect on recovery of MDH activity would parallel the effect on MDH binding. This was, in fact, observed when mutants with four, three, or two wild-type subunits in consecutive or nonconsecutive arrangements were examined (Figure 7, top, middle, and bottom panels, respectively). Full recovery was observed with three or four consecutive wild-type subunits, but much reduced recovery was observed with two consecutive units. Likewise, when three or four wild-type subunits were nonconsecutively arranged, the recovery of MDH was substantially reduced. We conclude that, as in the binding experiment, full recovery of native protein required the same three or more consecutive wild-type apical domains.

GroEL-Bound Rubisco Can Be Oxidatively Cross-Linked to Multiple GroEL Apical Domains

The foregoing experiments indicate a requirement for multiple binding-proficient apical domains to enable efficient binding of substrate polypeptides. To determine whether this reflects direct physical interaction with multiple apical domains, a cross-linking experiment was carried out, examining disulfide bond formation between cysteines in the substrate protein and a cysteine placed in each of the GroEL apical domains. A noncovalent GroEL variant, Cys261, was produced that has alanine substituted for the three endogenous GroEL cysteines per subunit and a cysteine replacing Thr261, an apical residue that points into the central cavity (Figure 2A). Cys261 functions normally both in vivo and in vitro (data not shown). A binary complex was formed between nonnative ^{35}S-labeled Rubisco diluted from acid (to 1

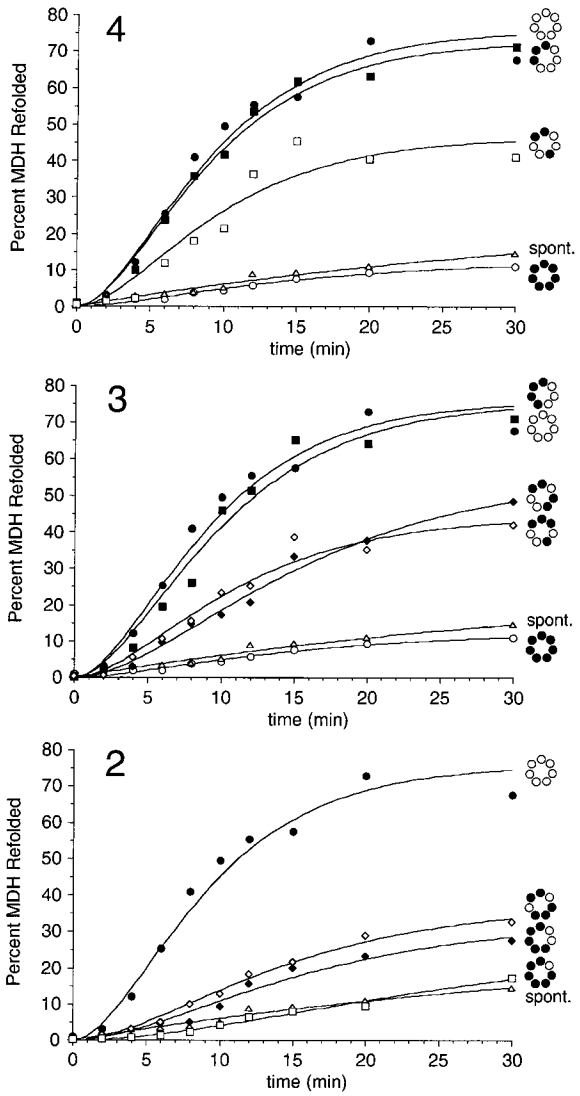


Figure 7. Folding of MDH Mediated by Mutant Complexes in the Complete Chaperonin System

MDH unfolded in guanidine-HCl was diluted into buffer containing the indicated chaperonin complex (designations as in Figure 2B), and percent of MDH refolded was determined by MDH enzymatic assay at the indicated time points. Top panel, arrangements of four wild-type subunits per ring; middle panel, arrangements of three wild-type subunits per ring; and bottom panel, arrangements of two wild-type subunits per ring.

μM) and Cys261 (2 μM) and subjected to air oxidation (upon removal of reductant by rapid gel filtration). Oxidation was halted by blocking unreacted cysteines with an excess of N-ethylmaleimide, and the reaction products were fractionated in a nonreducing 6% SDS gel (Figure 8). Using the covalent GroEL molecules with various numbers of subunits as size markers (Figure 1), we interpreted the pattern of the autoradiograph to indicate linkage between nearly all of the input Rubisco and from one to five GroEL subunits (Figure 8). At the migration positions of one, two, and three GroEL subunits, multiplets were observed around the appropriate migration position, presumably reflecting different branched structures formed by cross-linking of Cys261 subunits to various cysteines of the five present along the Rubisco polypeptide. The distribution of cross-linked Rubisco among multiples of GroEL subunits in these experiments was 27%, $GroEL_1$; 29%, $GroEL_2$; 25%, $GroEL_3$; 15%, $GroEL_4$; and 4%, $GroEL_5$. Thus, 73% of cross-linked molecules were cross-linked to more than one GroEL subunit. The percentage cross-linked to three or more, corresponding to the binding requirement observed in the mutant study, was 44%.

Consistent with this interpretation, when the same experiment was carried out with a Rubisco variant, C58A, which contains only four cysteines, a similar pattern was observed, except that the largest species was now absent (lane 8), presumably reflecting inability of this polypeptide, in the absence of a fifth cysteine, to form a cross-link with a fifth GroEL subunit. When the order of addition was reversed, adding iodoacetamide to alkylate the cysteines of Cys261 before polypeptide, no cross-linked species were observed (lane 3), indicating that it was the apical cysteines of Cys261 that were involved in producing the species cross-linked to radioactive Rubisco. Likewise, if an excess of nonradiolabeled Rubisco was added along with the radiolabeled protein during binary complex formation, the amount of subsequently cross-linked species was substantially reduced (lane 5), indicating that cross-linking took place from Rubisco bound in the central cavity, competed for by nonlabeled Rubisco. Furthermore, if a binary complex was formed first between nonradiolabeled MDH and Cys261, before addition of radiolabeled Rubisco, no cross-linked radioactive species were observed (lane 6). In an experiment examining productivity of the disulfide-linked molecules, disulfide cross-linked Rubisco-Cys261 complexes were exposed to DTT to reverse the cross-links (as in lane 2), and GroES and ATP were added to the mixture. This produced quantitative recovery of Rubisco enzyme activity, indicating that Rubisco cross-linked to Cys261 occupied a conformation capable of reaching the native state.

The length of the air oxidation reaction, typically <5 min (23°C), raised concern that cross-linking might not be occurring simultaneously but sequentially, with an initial disulfide cross-link tying the molecule down and facilitating formation of the next cross-link. While this cannot be rigorously excluded, particularly if bound polypeptide is mobile on a short timescale, reaction times as short as 1 min could be employed without a change in the pattern or its distribution. Oxidation time could be shortened to a few seconds by the use of a Cu(II)-phenanthroline catalyst (Careaga and Falke, 1992), producing the same pattern (data not shown). Here, however, we observed with Cys261 alone that more than two GroEL subunits could cross-link to each other (data not shown), raising concerns that non-thiol cross-links were being formed via oxidative byproducts. In contrast, no such cross-links, other than between two GroEL subunits (presumably through their unique cysteines), were formed with air oxidation. We conclude that, at least within the time limits of the present experiment, Rubisco exhibited physical association with multiple GroEL apical domains.

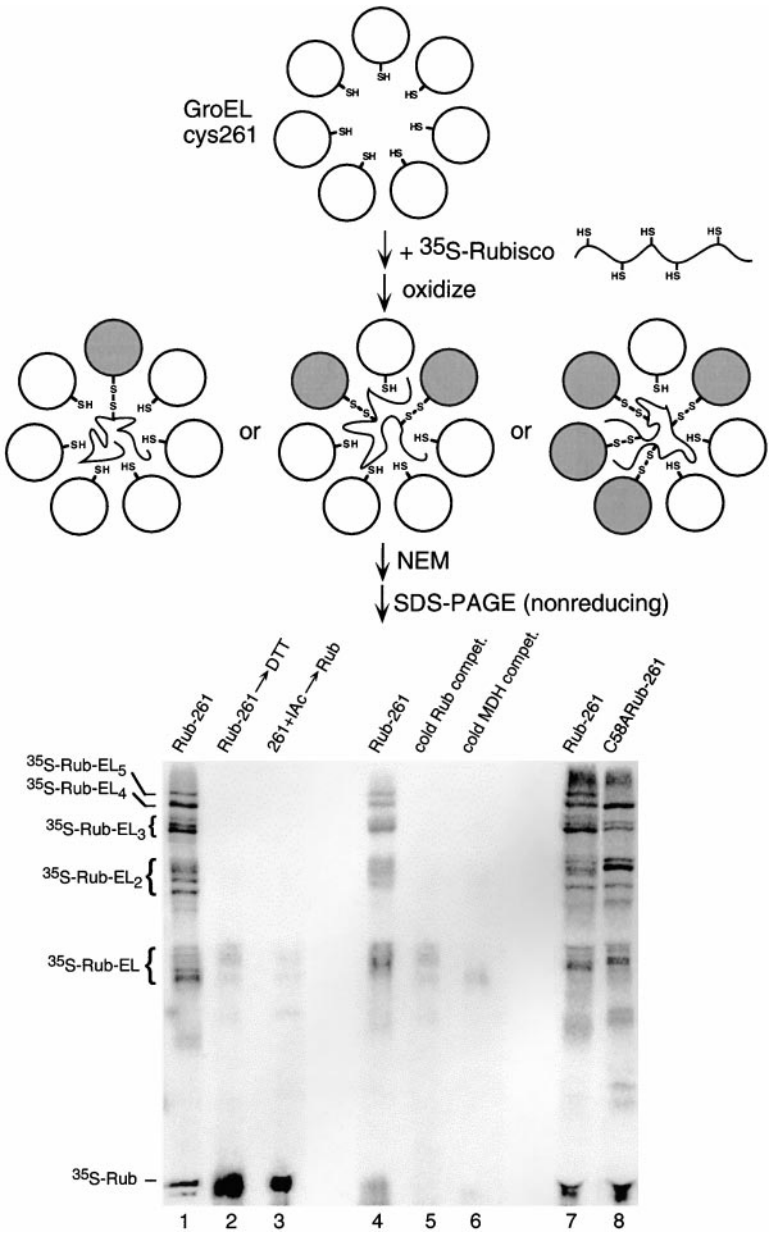


Figure 8. Oxidative Cross-Linking of Rubisco to Cys261 GroEL in a Binary Complex

Binary complexes of ^{35}S-Rubisco and Cys261 GroEL were subjected to air oxidation upon removal of DTT by gel filtration. The oxidation reaction was quenched by adding NEM, and the samples were fractionated on a 6% SDS-PAGE gel in the absence of reducing agent and analyzed using a Phosphorimager. Three panels showing individual experiments are presented. For each experiment, the first lane shows cross-linked products of the reaction as described (lanes 1, 4, and 7). The various species are labeled in the lefthand margin, with non-cross-linked Rubisco at the bottom of the gel and GroEL-cross-linked species above it. Subscripts indicate the number of GroEL subunits that have become cross-linked to Rubisco as judged by molecular size relative to nonlabeled covalent GroEL molecules on the Coomassie-stained gel (data not shown). Note multiplet of species for $GroEL_1$, $GroEL_2$, and $GroEL_3$, presumed to be produced by the branched character of various products, resulting in migration differences. Lane 2, reaction products treated with DTT prior to SDS-PAGE; lane 3, Cys261 complex treated with iodoacetamide prior to addition of nonnative ^{35}S-Rubisco; lane 5, 14-fold excess of nonlabeled Rubisco added at the same time as ^{35}S-Rubisco during binary complex formation; lane 6, binary complex of MDH-Cys261 formed prior to addition of ^{35}S-Rubisco; and lane 8, variant form of Rubisco, C58A, containing only four cysteines, used instead of wild-type ^{35}S-Rubisco.

Discussion

Binding of Nonnative Substrate Proteins through Multiple Apical Domains

The experiments reported here provide both functional and physical evidence that binding of three substrate proteins, Rubisco, MDH, and rhodanese, by GroEL is multivalent, involving interaction of substrate protein with multiple apical domains of the seven-membered GroEL ring. These proteins are stringent substrates; that is, they are dependent on the complete chaperonin system (GroEL, GroES, and ATP) to reach native form following dilution from denaturant (Goloubinoff et al., 1989; Schmidt et al., 1994; Ranson et al., 1995). Without an initial binding to GroEL, they misfold and aggregate in the bulk solution and, as a result, achieve no significant recovery of the native state. Rhodanese is considered to be somewhat less stringent than the others because it can be refolded, albeit less efficiently, when ADP is substituted for ATP (e.g., Weissman et al., 1996). The functional studies here tested the ability of mutant GroEL complexes, bearing different numbers and arrangements of apical domain–substituted subunits, to bind the substrate proteins when they were diluted from denaturant. These studies demonstrated a requirement for a minimum of three consecutive wild-type subunits to obtain a normal extent of binding of Rubisco and MDH, and for two wild-type subunits in any arrangement for rhodanese. When refolding of MDH was studied with the complete chaperonin system, i.e., in the presence of mutant GroEL complex, GroES, and ATP, the extent of recovery of the native state exactly paralleled the extent of MDH-GroEL binary complex formation. This effect on recovery of the native state would be expected

for the situation where failure of substrate to become bound to the open GroEL ring leads ultimately to irreversible misfolding and aggregation.

The results of functional studies in vivo resembled those of the in vitro experiments with the two stringent substrates, with rescue of GroEL-deficient cells requiring constructs with at least four consecutive wild-type subunits. Here, the requirement for an additional wild-type subunit may be due to the presence of covalent connections between subunits within the ring, which were observed in in vitro studies to reduce the extent of binding. Thus, both stringent substrate proteins in vitro and those endogenous *E. coli* proteins most dependent on GroEL in vivo require multiple functional apical domains for efficient binding and folding.

The functional evidence for involvement of multiple apical domains in binding stringent substrates was corroborated by the physical evidence that ~60% of GroEL-bound Rubisco molecules formed disulfide cross-links to two or more GroEL apical domains bearing a cysteine in the polypeptide binding surface. While oxidative cross-linking likely relies on a degree of mobility of the bound nonnative substrate to allow alignment of its cysteines with those in the apical domains, the observation that reversal of the cross-links was followed by productive folding (upon addition of GroES and ATP) indicates that the conformational states that become multivalently cross-linked probably lie within the collective of physiologically relevant ones.

Contiguous versus Noncontiguous Binding Sites

The studies of binding of Rubisco and MDH by mutant complexes indicated a preference for consecutive wild-type apical domains within a ring as opposed to interrupted arrangements. This may reflect a need for a continuous hydrophobic binding surface by these substrates, but an alternative possibility is that a contiguous arrangement enables efficient cooperative adjustment of neighboring apical domains upon initial binding of substrate protein, which favors stable binding. Such an adjustment could be disfavored by interposition of mutant apical domains. To date, however, there is no compelling evidence for cooperative action of the apical domains in polypeptide binding. On the other hand, nonnative rhodanese binds equally well to any arrangement of two wild-type subunits. Whether this reflects the somewhat reduced stringency of this protein or the structure of the nonnative state that is bound is not clear.

Recent observations in cryo-EM of actin bound to the eukaryotic cytosolic chaperonin, CCT, whose rings comprise eight nonidentical subunits, reveal a contrasting situation in which a noncontiguous arrangement seems to be observed. Reconstructions suggest that nonnative actin binds specifically to either of two noncontiguous pairs of CCT subunits, arranged in positions 1 and 4 (clockwise or counterclockwise) relative to each other (Llorca et al., 1999). This particular geometry may be imposed by the combination of the inherent asymmetry of the CCT apical domains and the preferred conformation of the bound actin. The functional significance of this arrangement remains to be established.

Normal Affinity for GroES by Complexes Unable to Bind Polypeptide

The observation that complexes with only two wild-type apical domains could still bind GroES normally in the presence of ATP came as a surprise. Even a single wild-type apical domain allowed substantial affinity for GroES. This could be either a function of the apical substitution employed, or, alternatively, a more general reflection of the nature of GroES binding. While V263 lies directly in the center of the polypeptide binding surface of the subunits of an unliganded acceptor ring (Figure 2a), upon GroES binding, with the attendant 60° elevation and 90° clockwise rotation of the apical domains, V263 moves into a new hydrophobic interface formed between apical domains (Xu et al., 1997). Thus, in contrast with other binding surface residues such as L234, L237, and the neighboring residue, V264, which make direct contact with the mobile loop of GroES, V263 does not make such contact, and its role in GroES binding may be indirect, relating to the ability of the new interface to support the overlying direct contacts with GroES. Thus, V263S might only mildly affect GroES binding as a function of its position. On the other hand, the steep dropoff of binding that occurs when only a single wild-type subunit is present may reflect a cooperative role of the apical domains in binding GroES. Initial contact of GroES with only one or two wild-type apical domains may be sufficient to drive cooperative changes, here in the neighboring mutant apical domains, that direct efficient, high-affinity, binding of the 7-valent GroES ligand (normally K_d~1 nM). Studies with additional apical mutant complexes, as well as perhaps with monovalent GroES ligands, may be able to establish such behavior.

Multivalency and Chaperonin Function

The functional requirement for multiple binding-proficient GroEL subunits observed here may reflect further on why the chaperonin is organized as a ring structure. Not only does the GroEL ring provide the opportunity to form a GroES-enclosed *cis* folding chamber during the folding-active phase of the chaperonin cycle, but even during the binding phase of the cycle, interaction with multiple subunits of a ring provides both more stable and more productive complex formation. While several studies have examined activities of isolated apical domains, it seems clear that they are insufficient to mediate binding or folding of stringent substrates like those examined here. For example, even in the setting of an intact ring, a single wild-type subunit with six neighboring apical mutant subunits was unable to support binding of Rubisco, MDH, or even rhodanese (U1,2,4,5,6,7 263; Figures 4 and 5).

How the GroEL system uses multivalency to achieve efficient binding has not been resolved by the experiments reported here. The requirement for multiple binding-competent domains for the tested substrates could reflect either a passive effect of the amount of continuous hydrophobic surface necessary for stable binding or the more active partitioning of certain nonnative states onto the apical surface, or even chaperonin-directed unfolding to produce stably bound conformations. Additional data on the state of the bound substrate proteins will be necessary to distinguish among these

possibilities. The role of multivalency in folding, particularly in terms of the model that multivalent binding provides the opportunity for further unfolding of bound substrate proteins by "stretching on the rack" (Shtilerman et al., 1999), concomitant with GroES binding and large-scale apical domain movements, is also unresolved by the observations here. Dynamic experiments reporting the physical state of the folding proteins may be necessary to address these issues.

Experimental Procedures

Proteins

GroEL (noncovalent) and GroES, bovine rhodanese, and wild-type and site-directed mutant C58A versions of Rubisco from *R. rubrum* were expressed in *E. coli* and purified as previously described (Weissman et al., 1995; Rye et al., 1997). Pig heart mitochondrial malate dehydrogenase (MDH) was purchased from Roche. GroEL T261C (Cys261; noncovalent) was produced by site-directed mutagenesis of a GroEL coding sequence in which the endogenous cysteines at positions 138, 458, and 519 had been replaced with alanine (Rye et al., 1999). Cys261 GroEL was expressed and purified as above and stored in buffer A (50 mM Tris [pH 7.4] and 50 mM KCl) containing 5 mM DTT and 2 mM TCEP under argon at −80°C.

Covalent GroEL molecules were expressed in DH5α from *trc* plasmids containing the various GroEL coding sequence fusions (construction details available). Cultures were grown at 25°C in the absence of induction, monitored periodically to assure that only 7-mer molecules were present, and harvested at an OD_{650} of 1.2–1.6. Cells were lysed in a Microfluidizer (Microfluidics), and high-speed supernatants were fractionated by Fast Flow Q-Sepharose chromatography. The GroEL-containing fractions were subjected to "stripping" by incubation with Affigel blue matrix (Biorad) and then gel filtered on a Superose 6 column (Amersham Pharmacia Biotech) in buffer A. Purified complexes were stored at −80°C. Typically, 12 liters of culture yielded ~20–50 mg of purified covalent complex.

Proteolytic removal of connections between subunits was carried out by incubating 20 μM covalent GroEL in buffer A with 20 μg/ml proteinase K (Sigma) at 25°C for 10 min. Proteolysis was halted by addition of PMSF to 2 mM, and proteolytically treated complexes were purified by gel filtration on a Tosohaas G4000SW_{xl} column eluted with buffer A. Radiolabeled Rubisco, rhodanese, and covalent GroEL complex were produced by expression in medium containing ^{35}S-methionine and purified in the same manner as the unlabeled molecules.

Assays

Polypeptide Binding

^{35}S-labeled Rubisco (10 μM) was unfolded in 25 mM glycine-phosphate (pH 2.0) at 25°C for 5 min and then diluted 10-fold into buffer B (50 mM HEPES [pH 7.6], 5 mM KOAc, 10 mM $Mg(OAc)_2$, and 1 mM DTT) containing 2 μM GroEL complex. After 15 min at 25°C, the mixture was fractionated on a G4000SW_{xl} column, eluted with buffer A. Fractions migrating at the position of GroEL were pooled, and the amount of GroEL-associated ^{35}S-Rubisco was quantitated by liquid scintillation counting. MDH (150 μM) was unfolded in 100 mM Tris (pH 7.4), 3 M GuHCl, and 10 mM DTT at 25°C for 2 hr. Unfolded MDH was diluted 100-fold into 0.4 ml buffer A containing 10 mM DTT and 2 μM GroEL complex and then incubated at 25°C for 30 min. The mixture was fractionated on a Superose 6 column, eluted with buffer A supplemented with 2 mM DTT. The GroEL-containing fraction, typically ~1.5 ml, was acidified by addition of 60 μl 1M H_3PO_4 and MDH separated by reverse phase chromatography on a 1 ml C4 column (VYDAC) using a gradient of 30%–70% acetonitrile in 0.1% TFA. MDH was quantitated by integration of the 280 nm absorbance peak eluting at ~50% acetonitrile. ^{35}S-labeled rhodanese (100 μM) was unfolded in 100 mM Tris (pH 7.4), 6 M GuHCl, and 10 mM DTT at 25°C for 1 hr and then diluted 100-fold into buffer A containing 1 mM DTT and 2 μM GroEL. The mixture was incubated at 25°C for 30 min and then the amount of GroEL-bound ^{35}S-rhodanese was determined as described above for ^{35}S-Rubisco.

GroES Binding

The Hummel-Dreyer assay, measuring GroES binding to GroEL in the presence of ATP during gel filtration, was carried out essentially as described (Weissman et al., 1996), except that 0.5 nmol GroEL was applied to the column in running buffer containing 2 mM ATP.

MDH Refolding

Refolding of MDH by the various GroEL species was assayed as described previously (Ranson et al., 1995), using MDH diluted from GuHCl as above, to 1.5 μM, 2 μM GroEL complexes, 4 μM GroES, and 2 mM ATP.

Stability of Rings against Subunit Exchange during Proteolytic Treatment

Covalent GroEL (2 mg/ml) in buffer C (50 mM potassium phosphate [pH 8.0] and 150 mM KCl) was labeled with biotin, using 1 mM sulfo-NHS-biotin (Pierce) and incubation at 0°C for 2 hr. Excess cross-linker was removed by four sequential 100-fold dilutions with buffer D (50 mM Tris [pH 7.4] and 150 mM KCl) and concentration using a Centricon 30 (Millipore). The biotinylated covalent GroEL was further purified using Ultralink monomeric avidin (Pierce), according to the supplier's recommendations. Exchange of GroEL subunits was assayed by digesting a mixture of 10 μM biotinylated covalent GroEL and 10 μM ^{35}S-labeled covalent GroEL with proteinase K as described above. A 20 μl aliquot was diluted to 200 μl with buffer D and incubated with 200 μl of a 1:1 suspension of Ultralink monomeric avidin resin in buffer D at 4°C for 2 hr. The supernatant was recovered for analysis; the resin was washed five times with 0.5 ml of buffer D and then eluted with 0.3 ml buffer D supplemented with 10 mM biotin. Samples were analyzed in a 6% SDS-PAGE gel, which was stained with Coomassie Blue, and then quantified by Phosphorimager detection of ^{35}S.

Disulfide Cross-Linking of Rubisco-Cys261 Binary Complexes

Binary complexes of ^{35}S-Rubisco and Cys261 GroEL were formed as described above, with final concentrations of 1 μM Rubisco and 2 μM Cys261, and purified using a PD-10 column (Amersham Pharmacia Biotech), eluted in buffer A. This step removed DTT and permitted rapid and gentle air oxidation. Further disulfide bond formation was quenched by adding 40 mM N-ethylmaleimide (NEM). Samples were concentrated using a Centricon 30 and prepared for SDS-PAGE by heating at 90°C for 1 min in SDS-PAGE sample buffer containing 40 mM NEM but no reducing agent. When iodoacetamide treatment of Cys261 GroEL was carried out before binary complex formation, the chaperonin (0.5 μM) was first incubated for 18 hr in 0.7 mM iodoacetamide at 4°C and then excess iodoacetamide was removed by gel filtration on a PD-10 column eluted with buffer A. The alkylated chaperonin was concentrated using a Centricon 30, and binary complexes were formed and processed as described above. For competition with cold Rubisco, 1 μM ^{35}S-Rubisco and 14 μM unlabeled Rubisco were unfolded together in acid. This mixture was used as substrate for disulfide cross-linking as above. When MDH was bound as a competitor to Cys261, it was unfolded and diluted into buffer containing chaperonin as described above (with final concentrations of both MDH and Cys261 complex of 1.5 μM). After 30 min at 25°C, unfolded ^{35}S-Rubisco (1 μM) was diluted 10-fold into the mixture followed by a 15 min incubation. The sample was then applied to a PD-10 column and processed as described above.

Cryo-EM

Approximately 1000 views each of the covalent heptamer, cleaved heptamer, wild-type GroEL, and V263S (noncovalent) GroEL were collected on a JEOL 1200 EX microscope with an Oxford Instruments cryotransfer stage. Films were digitized on a Leafscan 45 film scanner with a 10 μm step size, and the sampling was reduced to 6.6 Å/pixel. Starting models were obtained by 7-fold back projection of averaged side views, and the maps were refined by projection matching using SPIDER (Frank et al., 1996; Roseman et al., 1996). The resolution was determined by Fourier shell correlation.

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