Role of the GroEL Chaperonin Intermediate Domain in Coupling ATP Hydrolysis to Polypeptide Release*

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Modification of the Escherichia coli chaperonin GroEL with N-ethylmaleimide at residue Cys138 affects the structural and functional integrity of the complex. Nucleotide affinity and ATPase activity of the modified chaperonin are increased, whereas cooperativity of ATP hydrolysis and affinity for GroES are reduced. As a consequence, release and folding of substrate proteins are strongly impaired and uncoupled from ATP hydrolysis in a temperature-dependent manner. Folding of dihydrofolate reductase at 25 °C becomes dependent on GroES, whereas folding of typically GroES-dependent proteins is blocked completely. At 37 °C, GroES binding is restored to normal levels, and the modified GroEL regains its chaperone activity to some extent. These results assign a central role to the intermediate GroEL domain for transmitting conformational changes between apical and central domains, and for coupling ATP hydrolysis to productive protein release.

The Escherichia coli chaperonin GroEL plays a pivotal role in the folding of proteins in the cell. Assisted by the cofactor GroES, it allows newly synthesized polypeptide chains to gain their native state under the crowded conditions of the cytosol (for review, see Refs. 1 and 2). GroEL forms a cylinder composed of two symmetrically stacked rings, each with seven identical Mr 57,000 subunits (3, 4). Substrate proteins bind to the interior surface of this cylinder at the level of the apical domains (5, 6). Typically, only one substrate protein binds to one GroEL tetradecamer (5, 7). The conformation of substrate protein in its GroEL-associated state has been analyzed by tryptophan fluorescence, binding of the hydrophobic fluorescent dye anilino naphthalene sulfonate, and hydrogen-exchange experiments coupled with mass-spectrometry and NMR (7–12). According to these results, the bound proteins are in a "molten globule"-like folding state, marked by the presence of secondary structure, lack of persistent tertiary interactions, and the exposure of hydrophobic structure elements at the protein surface. GroEL has a relatively weak ATPase activity which is regulated by GroES (7, 13). This smaller cofactor is comprised of seven Mr 10,000 subunits that are arranged in a single ring. GroES can bind on top of the GroEL cylinder in the presence of nucleotides. A result of this interaction is an increased nucleotide affinity of GroEL and a tight coordination of the cycles of ATP binding, ATP hydrolysis, and ADP release.

The mechanism of GroEL/GroES-mediated protein folding has been subject of extensive investigation. The current data can be summarized in a model (1, 2), according to which ATP hydrolysis in the GroEL ring that is opposite GroES leads to release of ADP in the GroES-containing ring, accompanied by transient GroES release and binding of ATP. Once ATP is bound, GroES caps the end of the GroEL cylinder that also contains the substrate protein, thereby discharging the unfolded polypeptide into the shielded cavity for folding. Subsequent ATP hydrolysis in the opposite GroEL ring leads to the release of GroES and allows a folded substrate protein eventually to exit the cylinder. However, polypeptides that fold incompletely and still expose binding sites for the chaperonin after one such reaction cycle, will rebind. Typically, a polypeptide has to undergo several reaction cycles in association with GroEL before it leaves the chaperonin as a native or native-like protein (14–16).

The crystal structures of GroEL, GroES, and complexes between these two proteins have been solved (17–19), indicating the positions where nucleotides and the unfolded substrate proteins bind. Binding elements for GroES and polypeptide reside, and partially overlap, within the outer domains, whereas nucleotides bind to the central amino-terminal domains (7, 18). Electron microscopy studies, and the recently solved GroEL/GroES crystal structure have revealed the significant conformational changes that occur after nucleotide-dependent binding of GroES to GroEL (4, 19, 20). Most prominently, the apical GroEL domains move outward about 60°, leading to a significant widening and enlargement of the central cavity. These domain movements seem to be mediated by an intermediary hinge region (residues 134–190 and 377–408) which links the central and apical domains. In addition to its critical role in effecting these GroES-induced conformational changes, the hinge region is also thought to be involved in mediating the changes in the central ATP-binding region which are induced by the binding of substrate protein in the apical domain. Conversely, nucleotide binding and ATP hydrolysis in the central domain affect binding and release of substrate protein from a distance, and the intermediary domain is expected to be involved in this process as well.

We have previously observed that modification of GroEL with NEM1 leads to structural changes in the intermediary hinge region and, unlike wild-type GroEL, makes it accessible to attack by proteinase K (21). In this study, Cys138, a residue located in the intermediate domain, is identified as the target for NEM modification. As it seemed likely that NEM modification not only affects the structure, but has even more profound

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1 The abbreviations used are: NEM, N-ethylmaleimide; [α-32P]-8-N3ADP and [α-32P]-8-N3ATP, azido-labeled, radioactive adenosine diphosphate and adenosine triphosphate, respectively; DHPH, dihydrofolate reductase; MOPS, 3-(N-morpholino)propanesulfonic acid; GdnCl, guanidinium chloride; PAGE, polyacrylamide gel electrophoresis; ATPγS, adenosine 5′-O-(3-thiotriphosphate).

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effects on the function of the GroEL chaperonin, this study analyzes the consequences of NEM modification for the ability of GroEL to successfully mediate the folding of substrate proteins.

**EXPERIMENTAL PROCEDURES**

**Construction of GroEL Mutants**—Mutations in GroEL were generated in the plasmid pOF39 (22) by insertion mutagenesis and polymerase chain reaction. The following sequence changes were introduced: Cys138 → Ser (TGC → TCC); Cys458 → Ser (TGC → TCT); Cys519 → Ser (TGC → TCC). To replace the wild-type sequence with the mutated versions, the polymerase chain reaction fragments were digested with 

MnlI/HpaI (Cys138), DraIII/Nsal (Cys458), and DraIII/SmaI (Cys519). All constructs were finally sequenced to verify the intended changes. Standard genetic and molecular biology techniques were performed according to published protocols (23) or the manufacturers’ instructions.

**Protein Purification and Modification**—GroEL and GroES were isolated from an overproducing E. coli strain harboring plasmid pOF39 as described (7, 8, 22). 3H-GroES was prepared by reductive methylation (4). Modification of GroEL with NEM (2 mM) was performed as published (21). After modification, proteins were dialyzed against buffer A (50 mM MOPS, pH 7.2, 50 mM KCl, 5 mM MgCl₂).

**Refolding Experiments**—For refolding studies, enzymes were denatured in 5.3 M GdmCl, 2 mM dithiothreitol for 1 h at 25 °C. Denatured DHFR (5.4 μM), male dehydrogenase (66 μM), or rhodanese (80 μM) were diluted 120-fold into a solution containing a 2-fold molar excess of GroEL or NEM-GroEL in buffer A plus 5 mM dithiothreitol, and for rhodanese also 50 mM sodium thiosulfate. A 3-fold molar excess of GroES over GroEL was present as indicated. Folding reactions that were performed at 25 or 37 °C were initiated by addition of 3 mM ATP. The extent of reactivation was determined by monitoring the restoration of enzymatic activity at defined time points using established assays (7, 14, 24).

**Binding of GroES to GroEL and NEM-GroEL**—1 μM 3H-GroES was added to 0.25 μM GroEL or NEM-GroEL in buffer A plus 1 mM dithiothreitol and 1 mM ADP. Samples were incubated for 10 min at 25 or 37 °C, and excess unlabeled GroES was added at different concentrations for an additional 15 min, followed by analysis on native PAGE and fluorography (4). Bands corresponding to chaperonin complexes containing 3H-GroES were quantitated by laser densitometry.

**Miscellaneous**—Cross-linking of [α-32P]8-N₃-nucleotides (ICN) to GroEL (30), GroEL ATPase assays (8, 26), determination of Hill coefficients (25, 27), measurement of rhodanase aggregation by light scattering (7), and tryptophan fluorescence measurements of DHFR folding intermediates (7) were all performed according to published procedures.

**RESULTS**

**Modification of Cys³²⁸ Affects Nucleotide Binding and ATP Hydrolysis in NEM-GroEL**—Previous studies have shown that after treatment of GroEL with NEM, the modified chaperonin complex (NEM-GroEL) becomes protease-sensitive. Incubation of wild-type GroEL with protease K in the presence of MgADP or MgATP results in the removal of the 16 carboxyl-terminal residues from all 14 GroEL subunits (4, 14). In NEM-GroEL the region around residues 142–152 also becomes accessible to protease resulting in cleavage of the subunit into an amino-terminal M₁, 15,000 and a carboxyl-terminal M₂, 40,000 fragment (21). These fragments correspond to the structural and morphological domains seen in electron micrographs and the crystal structure (4, 17, 18). In the absence of nucleotides, NEM-GroEL is completely digested by protease. The intermediary domain, to which protease K gains access in NEM-GroEL, is thought to transmit conformational changes upon nucleotide binding from the upper part of the central domain to the outer apical domain, where substrate protein and GroES bind, and vice versa. It was therefore of interest to find out whether in NEM-GroEL nucleotide binding at ATP hydrolysis is affected by the modification and whether, consequently, the communication between the central and apical domain(s) is disturbed. In fact, the ATPase activity of NEM-GroEL was found to be strongly increased in comparison to wild-type GroEL (Fig. 1A). Although GroES, which reduces the ATPase rate in wild-type GroEL to about 50%, exerts a comparable effect also on NEM-GroEL, the absolute ATPase rate of the modified chaperonin is still as high as that of wild-type GroEL in the absence of GroES. Binding of unfolded substrate proteins to the chaperonin is known to stimulate the GroEL ATPase (7, 14, 28–31), indicating that binding of unfolded proteins induces conformational changes in GroEL, which affect nucleotide binding and the rate of ATP hydrolysis. Surprisingly, the exact opposite effect was observed when unfolded substrate protein was added to NEM-GroEL. The rate of ATP hydrolysis was reduced by a factor of 3 (Fig. 1A). Effects of the NEM modification on the apparent affinity of the chaperonin for nucleotides were analyzed by measuring the extent of covalent labeling of GroEL and NEM-GroEL with [α-32P]8-

![Fig. 1. ATPase activities and nucleotide affinities of GroEL chaperonins.](http://www.jbc.org/)
N₃ADP and [α-³²P]8-N₃ATP bind specifically to GroEL, and the ATP analog can be hydrolyzed efficiently (14, 25). The use of these analogs for functional studies has allowed us previously to define important aspects of the chaperonin mechanism. The specificity of azidonucleotide labeling was demonstrated by the identification of Tyr⁷⁸ as a residue within the nucleotide-binding region in GroEL (18, 25). As expected, binding of both azido-ATP and azido-ADP to wild-type GroEL were increased in the presence of GroES, whereas addition of substrate protein led to a reduced binding of nucleotide (Fig. 1B). NEM-GroEL displayed a higher affinity for nucleotide than wild-type GroEL but, unlike with wild-type GroEL, in the presence of substrate protein binding of nucleotides was further increased. GroES addition did not lead to a strong additional increase in nucleotide binding.

GroEL contains three cysteine positions in 138, 458, and 519. Cys¹³⁸ lies in a relatively exposed loop in the intermediary domain (17). The fact that this corresponds exactly to the position where cleavage by proteinase K occurs after NEM modification (21) makes Cys¹³⁸ an obvious candidate for being the target of the modifying agent. The other two cysteines occupy rather buried positions within the chaperonin complex, as is evident from the GroEL crystal structure (17). To determine which of the three residues is responsible for the altered behavior of NEM-GroEL, the cysteines were individually exchanged to serine residues. Earlier results have shown that these replacements result in functional proteins, although their effectiveness in reactivation of certain substrate proteins and ATPase rates is somewhat reduced (32–34). However, for the purpose of identifying the critical cysteine residue(s), only relative ATPase rates of these mutants in comparison to each other had to be considered here. Mutants were assayed for their ATPase activities, with or without prior NEM modification. Whereas wild-type GroEL and the Cys⁵¹⁹ → Ser and Cys⁵⁰⁹ → Ser mutants displayed the expected increases in ATPase activities after their modification with NEM, exchange of the first cysteine in position 138 abolished sensitivity of the chaperonin toward NEM (Fig. 1C). This is consistent with Cys¹³⁸ being the residue that becomes modified by NEM. Supportive evidence for this conclusion comes from mass spectrometry analysis of the amino-terminal M₁₅₀₀₀ fragment originating from a proteolytic digest of NEM-GroEL, which showed a mass increase corresponding to one attached NEM moiety.² The two carboxyl-terminal cysteines are much less susceptible to NEM modification, and partial modification is only obtained at higher NEM concentrations than those used in this study.

**Temperature-dependent Folding Defects**—Properly regulated ATP hydrolysis is essential for a fully operational GroEL/GroES reaction cycle. To investigate whether the above described changes in NEM-GroEL had an effect on the ability of the chaperonin to efficiently release and refold substrate proteins, reactivation of the monomeric enzyme DHFR was analyzed. DHFR can refold spontaneously after dilution from GdmCl into buffer solution. Complexes of the chaperonin with DHFR were formed by dilution of the GdmCl-unfolded protein into buffer containing either GroEL or NEM-GroEL. NEM-GroEL was able to bind the protein with the same affinity as wild-type GroEL, as demonstrated by its ability to prevent the spontaneous reactivation of DHFR (not shown). Evidently this aspect of chaperonin function is not affected by the modification.

In a complete GroEL/GroES reaction cycle, the bound substrate protein is released into the chaperonin cavity for productive folding (14–16). On the other hand, for proteins that have the ability to fold spontaneously, ATP hydrolysis alone in the absence of GroES is already sufficient to release them from GroEL, albeit in a non-native state. Under these conditions, folding does not take place in the chaperonin cavity, but eventually occurs in solution. When refolding of DHFR at 25 °C was initiated by addition of ATP to a wild-type GroEL-DHFR complex in the absence or presence of GroES, the substrate protein regained enzyme activity efficiently in both cases (Fig. 2). In contrast, significant defects became apparent when NEM-GroEL was used as a chaperonin. Almost no reactivation took place in the absence of GroES, indicating that DHFR was unable to fold with ATP alone (Fig. 2). Interestingly, refolding proceeded quite efficiently when GroES was added to the reaction. Apparently, the structural changes in GroEL that were caused by NEM turned DHFR into a GroES-dependent chaperonin substrate. A different picture was observed when the incubation temperature was raised from 25 to 37 °C. At the higher temperature, NEM-GroEL-mediated reactivation of DHFR occurred with ATP alone in the absence of GroES (Fig. 2). The modification by NEM thus results in a “cold-sensitive” defect of the GroEL folding machinery.

**ATPase Activity in NEM-GroEL at 37 °C**—In accordance with a significant restoration of chaperonin activity at 37 °C, the suppressing effects of substrate protein on the ATPase activity of NEM-GroEL were less pronounced at the higher temperature (Fig. 3). Remarkably, however, the absolute rate of ATP hydrolysis of NEM-GroEL was still more than 2-fold higher than that of wild-type GroEL, arguing that the strongly increased ATPase activity of NEM-GroEL at 25 °C was not the major cause for the observed defects. It seemed possible that concomitant with the changes in nucleotide binding and ATP hydrolysis, the cooperativity of ATP hydrolysis was also affected in NEM-GroEL. Uncoordinated ATP hydrolysis of the individual GroEL subunits could lead to unproductive release of substrate proteins and could be a cause for the failure of NEM-GroEL to promote correct protein folding. Analysis of the cooperativity of initial ATP hydrolysis in NEM-GroEL in the presence of GroES revealed that it was significantly diminished in comparison with wild-type GroEL, not only at 25 °C, but also at 37 °C. Hill coefficients (n_H) of 1.02 at 25 °C and 1.12 at 37 °C were determined (see “Experimental Procedures” for details). In contrast, wild-type GroEL values of 2.58 and 2.31, respectively, were obtained.

**Uncoupling of ATP Hydrolysis from Efficient Polypeptide Release**—Two factors thought to be important for the produc-

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² C. Robinson, S. Radford, and J. Martin, unpublished results.
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...DHFR shows a Trp-emission spectrum that in intensity release-rebinding cycles of substrate proteins translate into productive protein folding. How would such a pattern of rapid state association of substrates with NEM-GroEL and preclude rapid rebinding. Such rapid cycling would result in steady-explained by an uncoordinated substrate release followed by irreversible binding of the unfolded polypeptides but could be exceeded to fold to the native form in solution. The inability of NEM-GroEL, the DHFR folding intermediates then proceed to fold to the native form in solution. The inability of NEM-GroEL to release substrate proteins is thus not due to irreversible binding of the unfolded polypeptides but could be explained by an uncoordinated substrate release followed by rapid rebinding. Such rapid cycling would result in steady-state association of substrates with NEM-GroEL and preclude productive protein folding. How would such a pattern of rapid release-rebinding cycles of substrate proteins translate into their tryptophan fluorescence spectra? It is known that GroEL-bound DHFR shows a Trp-emission spectrum that in intensity and $\lambda_{max}$ is intermediate between those of native and unfolded forms (Fig. 4B) (7, 26). It has the typical characteristics of a molten globule-like state. When ATP was added to the GroEL-DHFR complex, folding to the native state proceeded, signified by a rapid drop in fluorescence intensity which was followed by a slower blue-shift of $\lambda_{max}$ (7). A completely different picture emerged when an equivalent experiment was performed with a NEM-GroEL-DHFR complex. After addition of ATP, the Trp spectrum did not change at all. The curves taken after various time points fluctuated around the one representing the initial time point, and even after 35 min, a time when with wild-type GroEL folding had long been completed, the DHFR Trp spectrum appeared unchanged. It is important to note in this context that, although the ATPase activity of NEM-GroEL is diminished in the presence of bound substrate proteins (Fig. 1A), ATP hydrolysis is still going on. This observation corroborates the idea that the folding intermediate is either not released completely, or rebinds too rapidly before having the opportunity to internalize hydrophobic binding sites in the course of folding in the cavity.

Consequences of Impaired Interaction between NEM-GroEL and GroES—Although a lack of cooperativity in ATP hydrolysis by the individual chaperonin subunits may play an important role in the defects that became evident in NEM-GroEL (see above), the persistence of non-cooperativity at 37 °C shows that there must be yet another influential factor that is responsible for partially reversing the cold-sensitive phenotype. A possible candidate is the cofactor GroES. As indicated by its ability to reduce the NEM-GroEL ATPase activity by 50% (Fig. 1A), GroES can apparently interact with NEM-GroEL. However, the absolute rate of ATP hydrolysis is still considerably higher than that of a wild-type GroEL-GroES complex and it corresponds to that of wild-type GroEL in the absence of GroES. To get a more detailed picture of the actual strength of the NEM-GroEL-GroES interaction, $^3$H-GroES was incubated with GroEL or NEM-GroEL in the presence of ADP. Increasing amounts of unlabeled GroES were then added to the preformed complexes and the binary complexes were analyzed by native PAGE where GroEL-GroES complexes migrate as a distinct band. If $^3$H-GroES is bound tightly to GroEL, one would expect only little exchange with GroES to occur despite the presence of an excess of the unlabeled form. In fact, this was the result obtained with GroEL at 25 and 37 °C (Fig. 5). However, NEM-GroEL was observed to have a significantly reduced affinity for the chaperonin cofactor at 25 °C resulting in rather rapid dissociation of $^3$H-GroES and its replacement by the competing unlabeled GroES. At 37 °C NEM-GroEL regains the ability to interact with its cofactor efficiently (Fig. 5), suggesting that high affinity binding of GroES is a critical parameter for allowing the chaperonin to fulfill its role in assisted protein folding satisfactorily. Only by tightly docking on top of the GroEL ring that already has bound substrate protein can GroES restrict access to and from the inside of the cylinder. A major consequence of this high affinity interaction is that substrate proteins are prevented from leaving the cavity after release from their binding sites at GroEL. The observation that the interaction of NEM-GroEL with GroES was weakened at 25 °C raised the possibility that refolding of GroES-dependent substrate proteins was also affected. To address this issue, reactivation of two established GroEL substrates was analyzed, namely that of the monomeric enzyme rhodanese and that of dimeric malate dehydrogenase (Fig. 6). For malate dehydrogenase, the function of the chaperonin is to assist the formation of folded monomeric subunits in the cylindrical cavity, which is then followed by chaperonin-independent assembly of the subunits in solution. NEM-GroEL was unable to assist the refolding of both enzymes at 25 °C (Fig. 6). When the folding experiments were performed at 37 °C, partial reactivation was observed for both rhodanese and malate dehydrogenase. This result corroborates the model that the ability of GroEL to productively mediate folding of substrate proteins correlates with its ability to bind GroES with high affinity. Under these conditions substrate proteins are confined to the cavity for folding and are prevented from diffusing into the bulk solution in an unfolded state.

DISCUSSION

Modification of GroEL with NEM changes the properties of the chaperonin profoundly, both structurally and functionally. Only one of the three cysteines in GroEL, Cys$^{138}$ is modified by NEM. The small intermediate domain (residues 134–190 and 377–408), in which this residue resides, joins the apical domain of a GroEL subunit with its large equatorial domain. It has been proposed that this intermediate segment allows a hinge-
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like opening and twisting of the apical domain about the common domain junction (Gly192 and Gly375), which is caused by nucleotide binding in the equatorial domains and GroES binding to the apical domains of one GroEL ring (19). As a result, the volume of the central GroEL chamber in the GroES-associated ring increases significantly, forming the cage in which protein folding proceeds. The now dome-shaped cavity is spacious enough to allow the enclosed protein to explore its folding pathway without direct interference from the chaperonin itself.

In addition to moving upward the apical domain becomes twisted. The affinity of GroEL for unfolded polypeptide is strongly reduced when GroES is bound in the presence of nucleotides because rotation of the apical domains about the hinge between the apical and intermediate domains then occludes most of the hydrophobic-binding regions in the intersubunit interface. The opening movement of the apical domain is made possible by a downward movement of the intermediate segment toward the central channel which occurs at the domain junction between intermediate and equatorial domain around residues Pro137 and Gly410 (19). Further evidence for the importance of that second hinge region comes from the present study showing that modification of Cys138 adjacent to Pro137 with a bulky NEM moiety interferes with this movement. Structural changes in the vicinity of Pro137 can explain not only the observations described here but also our findings from an earlier study (21) where we analyzed fragments of NEM-GroEL that were obtained after proteolysis in the presence of nucleotides. Cleavage by proteinase K occurred between residues 143 and 153. Whereas Pro137 is highly conserved in members of the chaperonin family, one encounters more variability in the adjacent residue. In addition to Cys, one frequently finds Val at this position, and mutations to other small uncharged residues like Ala or Ser result in functional proteins (32–34). However, when we attempted to mimic the NEM effect by exchanging Cys138 to the larger hydrophobic residues Trp or Phe, we found defects similar to that observed with the chemically modified chaperonin. It appears that only modification with bulky hydrophobic groups perturbs the structural integrity in the complex in such a way that proteinase K gains access to an otherwise inaccessible region.

The primary consequences of modification of the critical residue Cys138 in NEM-GroEL are changes in nucleotide binding, rate and cooperativity of ATP hydrolysis, and GroES binding. NEM-GroEL has an increased ATPase activity and binds nucleotides with higher affinity than wild-type GroEL. This phenotype is unique among the various GroEL mutants that have been generated so far in that none of these analyzed mutants is characterized by an accelerated rate of ATP hydrolysis (6); typically, the rate is reduced. In a GroEL/GroES complex the

3 J. L. Mark and J. Martin, unpublished results.
downward movement around Pro$^{137}$ impedes dissociation of ADP, locking the chaperonin complex in the nucleotide-bound state. It is conceivable that NEM-GroEL adopts a conformation that mimics part of this movement and thereby leads to an increase in nucleotide affinity. This conformational switch could bring Cys$^{338}$ closer to the nucleotide-binding site, explaining earlier data from cross-linking experiments with ATPyS that had placed Cys$^{338}$ in the vicinity of the ATP phosphate groups (32). Remarkably, despite its higher affinity for nucleotides, the affinity of NEM-GroEL for GroES is reduced compared with wild-type GroEL. This result demonstrates that a tighter binding of ATP or ADP to the chaperonin does not automatically correlate with better binding of the chaperonin cofactor as expected. As a consequence of the weak interaction of NEM-GroEL with GroES, the modified chaperonin is compromised in its ability to promote protein folding at 25 °C. Substrate proteins are likely to make contact with GroEL at several of the seven subunits in the cylindrical ring. A coordinated release of substrate from all these sites during ATP hydrolysis would give the protein time to partially fold within the cavity until the high affinity ADP state is regenerated. Without GroES, nucleotide binding and ATP hydrolysis by the individual GroEL subunits result in nonproductive release of folding intermediates into the bulk solution. For smaller proteins that can fold relatively easily, such as DHFR, release per se may be sufficient. In contrast, for those substrates that are more likely to engage in non-productive intermolecular interactions and are prone to misfolding, such as rhodanese, full coordination of the conformational changes in the individual subunits and the presence of GroES is required for a productive release into the chaperonin cavity. Unsynchroized release of substrate protein, as in NEM-GroEL, would shorten the time window for folding, because at any given time a binding site in NEM-GroEL would be competent for (re)binding the substrate. GroES binding to NEM-GroEL may facilitate coordinated release at least to such a degree that is sufficient for the release and folding of the weakly binding substrate DHFR, whose reactivation consequently becomes GroES-dependent. However, for tight binding substrate proteins like rhodanese, the GroES interaction with NEM-GroEL is still too weak to promote their efficient displacement into the cavity. At 37 °C, several effects may contribute to the partially restored chaperonin activity of NEM-GroEL. First, folding of substrate proteins is expected to occur with faster kinetics, which might enable the folding proteins to escape an untimely rebinding to NEM-GroEL. Second, the conformational changes induced by NEM modification may be less pronounced at 37 °C, or may affect the chaperonin conformation less than at 25 °C due to an increased flexibility of the chaperonin structure. Third, and most importantly, there is a strong improvement in GroES binding to NEM-GroEL at higher temperatures. A firmly anchored GroES is required to displace the substrate protein from its binding site at GroEL which partially overlaps with that of the smaller cofactor. In fact, it appears that this aspect of GroES function is the most critical one, more important than a precise regulation of the GroEL ATPase activity (35). High affinity binding of GroES to the apical domain after nucleotide binding to the central domain would be made possible by the intermediate domain, coupling not only these two events, but also ATP hydrolysis to productive protein release.

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