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Entering deeper into the mysteries of the GroEL–GroES nanomachine

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In the densely populated intracellular milieu, polypeptides are at constant risk of nonspecific interactions and aggregation, posing a threat to essential cellular functions. Cells rely on a network of protein folding factors to deal with this challenge. The Hsp60 family of molecular chaperones, which depend on ATP for function, stands out in the proteostasis network by a characteristic structure comprising two multimeric rings arranged back to back. This review provides an updated overview of GroEL, the bacterial Hsp60, and its GroES (Hsp10) cofactor. Specifically, we highlight recent breakthroughs in understanding the intricate folding mechanisms of the GroEL–GroES nanomachine and explore the newly discovered interaction between GroEL and the chaperedoxin CnoX. Despite considerable research on the GroEL–GroES system, numerous questions remain to be explored.

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Introduction

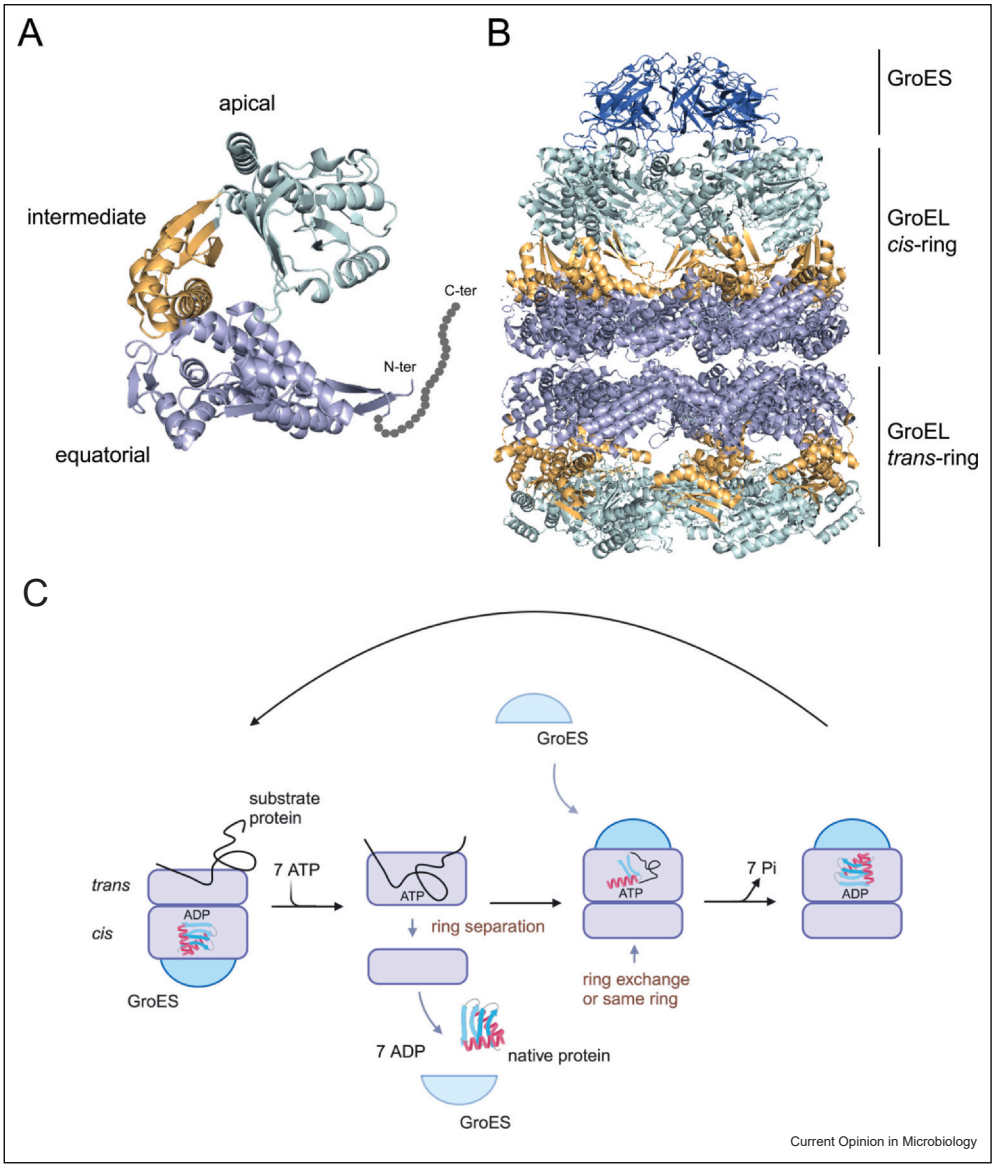
The intracellular space is characterized by macromolecular crowding, featuring cytosolic macromolecules at concentrations ranging from 300 to 400 g/L [1]. Because of that, polypeptides face the risk of nonspecific interactions and aggregation, not only during synthesis but also when subjected to stress-induced unfolding; aggregation of essential proteins may ultimately lead to the loss of cellular functions critical for growth and survival. Furthermore, protein aggregates can have cytotoxic effects by binding to and disrupting functional proteins [2]. In humans, protein aggregation is associated with cellular degeneration in many age-related diseases (such as Alzheimer's and Parkinson's diseases, among others) [3]. Therefore, controlling protein quality and maintaining proteome homeostasis are critical for cellular survival. To achieve this, living cells rely on a network of molecular chaperones that play key roles in processes such as *de novo* folding, refolding or proteolytic degradation of stress-denatured proteins, oligomeric assembly, and intracellular protein trafficking [4–7].

Protein quality control requires chaperonins

A distinct class of chaperones crucial for proteostasis in all kingdoms of life is the Hsp60 chaperone family [8]. Commonly known as 'chaperonins,' these chaperones, requiring ATP to function, exhibit a typical architecture with two multimeric rings, each featuring an inner central cavity, stacked back to back [8,9]. The Hsp60 family is categorized into two major groups [10]. Group I chaperonins are present in the bacterial cytoplasm (GroEL; see below), mitochondria (Hsp60), and chloroplasts (Cpn60), while group II chaperonins are found in archaea (thermosome) and in the eukaryotic cytosol (the main group II chaperonin is the protein tailless complex polypeptide 1 ring complex, also known as Chaperonin containing tailless complex polypeptide 1) [11,12]. A significant distinction between these two groups is that group I chaperonins require a detachable Hsp10 cofactor, which forms a multimeric dome-like structure, for their function. This Hsp10 lid is associated with Hsp60 in the presence of ATP, covering the apical opening of the chaperonin ring and creating a closed folding chamber where a substrate can be encapsulated [8,9]. In contrast, group II chaperonins do not require Hsp10; instead, they have a built-in structure that replaces the Hsp10 cofactor function [13].

Escherichia coli GroEL, a 57 kDa protein that assembles into two seven-membered rings, represents the archetypal member of the group I Hsp60 class [14–16]. Each subunit of GroEL can be divided into three domains (Figure 1). First, the interface between the two rings is formed by the equatorial domains, which also contain the ATP-binding site. The C-terminal residues of the equatorial domain of the different GroEL subunits form hydrophobic, intrinsically disordered tails that interact with substrate and are thought to block the hole between the rings [17–19]. Next, the intermediate hinge domain connects the equatorial domain to the flexibly attached apical domain. Together, the apical domains constitute the entrance to the cavity, exposing hydrophobic amino acid residues for substrate and Hsp10 binding [10,20]. The Hsp10 cofactor of GroEL is the protein GroES, an ~10 kDa protein that forms a heptameric lid (Figure 1) [21].

Figure 1



Structural and functional dynamics of the GroEL–GroES nanomachine. **(a)** Crystal structure of one GroEL subunit (PDB: 1SS8), showing the equatorial (slate), intermediate (orange), and apical domain (light cyan). **(b)** Crystal structure of the asymmetric GroEL–GroES nanomachine (PDB: 1AON; GroES is in dark blue). The GroES-bound ring is referred to as a *cis*-ring; the GroES-free ring is referred to as the *trans*-ring. **(c)** Mechanism of the GroEL–GroES nanomachine: the cycle initiates with the binding of a non-native substrate protein to the GroEL *trans*-ring. Subsequently, ATP molecules attach to the equatorial domain of this ring, allowing the transient separation of the GroEL double rings. GroES competes with the substrate for GroEL binding, resulting in the substrate being encapsulated within the folding chamber. After ATP hydrolysis occurs in the *cis*-ring, the cycle progresses with the binding of a new substrate and ATP molecules to the now-available *trans*-ring. GroES and ADP molecules are released from the *cis*-ring. PDB; Protein Data Bank.

GroEL and GroES, essential for growth and survival in *E. coli* [15], have been extensively studied for decades, with authoritative reviews dedicated to these proteins [8–10]. Here, while summarizing the most important features of the GroEL–GroES nanomachine, we will emphasize recent advances regarding its folding mechanisms and functional partners. Specifically, we will highlight a newly discovered, widely conserved protein-folding factor that assists GroEL–GroES in its function.

How does GroEL–GroES fold substrates?

A substantial body of research on the mechanism of the GroEL–GroES system supports a model in which the two rings of GroEL function sequentially. The cycle starts with the binding of a non-native substrate protein to the apical

domain of several GroEL subunits from a GroES-free ring (Figure 1). Seven ATP molecules subsequently bind to the equatorial domain of the same ring, priming it for GroES interaction [20,22,23]. When GroES interacts with GroEL, it competes with the substrate at the top of the apical domain for the binding interface, leading to the displacement of the substrate into the folding chamber. The GroES-bound ring, where large conformational rearrangements of the intermediate and apical domains double the size of the GroEL cavity, is referred to as a *cis*-ring. In this conformation, the cavity wall is covered with hydrophilic residues. On the other hand, the GroES-free ring is referred to as the *trans*-ring. In this asymmetric complex, the substrate is confined in a compartment where it can fold while GroEL hydrolyzes the ATP molecules. This hydrolysis process typically takes 2 to 7 seconds, depending on temperature [10]. Meanwhile, another substrate can bind to the open *trans*-ring on the opposite side of the nanomachine. ATP hydrolysis in the *cis*-ring destabilizes the *cis*-complex, leading to its dissociation upon ATP binding in the *trans*-ring [24]. The *cis*-ring then reverts to the *trans*-conformation. Folded substrates are released, while non-native polypeptides are quickly captured by the same or another GroEL molecule for a new folding cycle. Depending on the substrate, one or more cycles may be required to reach the native state. An important new insight into the GroEL–GroES mechanism was recently reported [25]; it was shown that due to inter-ring negative allostery upon ATP binding, the GroEL double rings separate and exchange *in vitro* at approximately the same rate as ATP turnover, suggesting that ring separation occurs *in vivo* during each round of the GroEL–GroES cycle. This transient ring separation likely prevents the formation of symmetric complexes in which both GroEL rings are closed by GroES, providing further support to the model that GroEL–GroES functions sequentially.

Only a subset of cellular proteins depends on GroEL–GroES for folding

In bacteria, when newly synthesized polypeptides exit the ribosome tunnel, they interact with trigger factor, a ribosome-bound chaperone [6]. Trigger factor's action is generally sufficient to assist in the folding of most proteins [26,27], particularly when they are small in size. However, longer polypeptides may require additional assistance and benefit from the ATP-dependent chaperone activity of the DnaK-DnaJ-GrpE system. Only a subset of slow-folding and aggregation-sensitive proteins, comprising approximately 10% of the cytoplasmic proteome — increasing to ~30% under heat stress — is subsequently transferred from DnaK-DnaJ-GrpE to the GroEL–GroES nanomachine for folding [28,29]. Therefore, GroEL–GroES serves as the downstream component in a sequential pathway of chaperone action.

GroEL–GroES substrates typically exhibit lower folding propensities and a higher tendency to aggregate. In *E. coli*, their molecular weights usually range between 20 and 70 kDa, enabling them to fit inside the folding chamber of the chaperonin. Based on their degree of dependence on GroEL–GroES for folding, these substrates were classified into four classes [29,30]; around 80 proteins have been identified as obligate substrates of this system [30–32]. These obligate substrates, occupying about 80% of the GroEL–GroES capacity, are predominantly metabolic enzymes. They are enriched in proteins with the $(\beta/\alpha)_8$ triosephosphate isomerase (TIM)-barrel fold [23] and include enzymes such as 4-hydroxy-tetrahydrodipicolinate synthase (DapA) and methionine adenosyltransferase (MetK) [30,32]. Upon depletion of GroEL, obligate substrates are prone to aggregate, with the Lon protease playing a vital role in degrading these aggregates [33].

An active folding model that implies partial unfolding

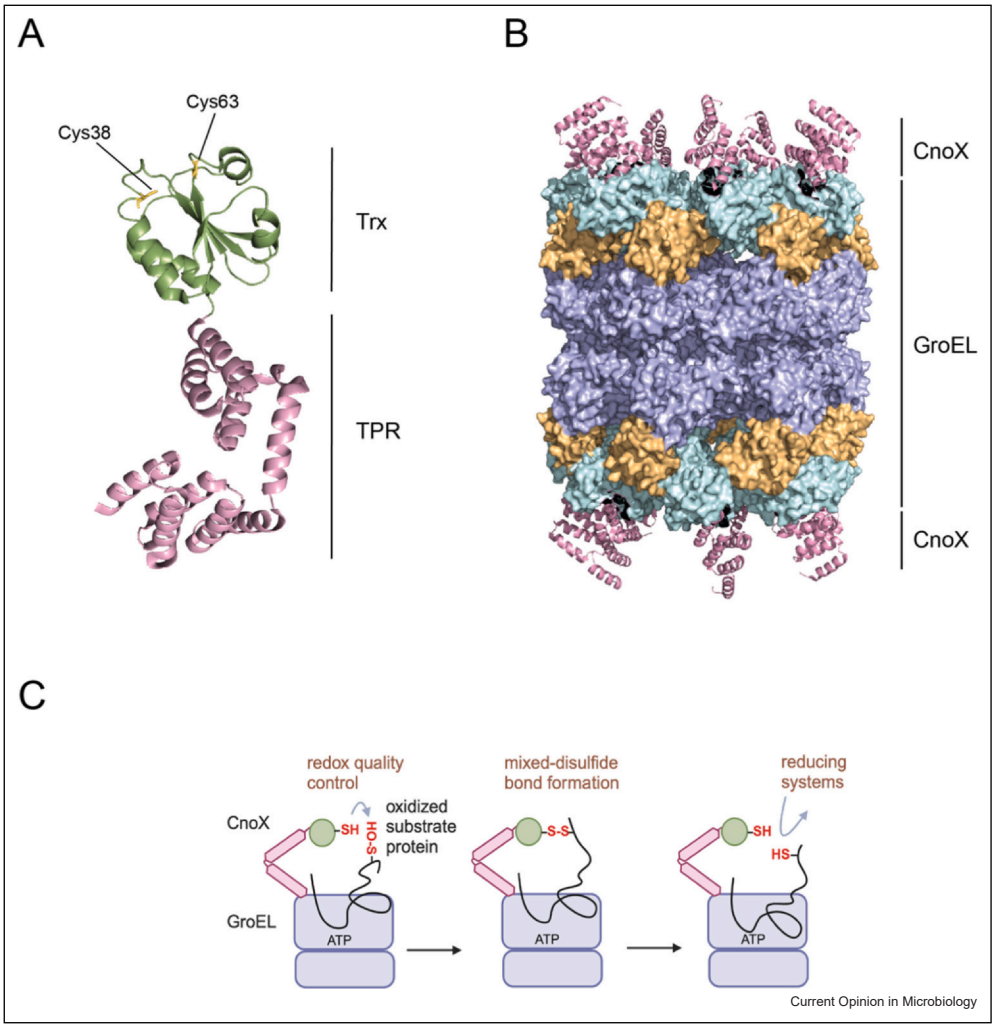
The mechanism through which GroEL–GroES promotes folding has been extensively investigated, either using model proteins [8], such as ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo) from *Rhodospirillum rubrum*, or endogenous substrates like the TIM-barrel fold protein DapA [34] and the metalloprotease PepQ [35]. While a comprehensive understanding of how GroEL–GroES facilitates substrate folding remains elusive, several key features contributing to this process have been identified. Firstly, it is believed that the binding of ATP in the equatorial region causes substantial conformational rearrangements of the apical domains, which in turn force the unfolding of the misfolded substrate bound to the apical domains. Recent structural insights from a series of GroEL complexes with a non-native RuBisCo revealed snapshots of this process and proposed a forced unfolding mechanism, where the substrate is stretched between the apical domains and the C-terminal tails of GroEL [36]. Interestingly, in one structure, four GroEL subunits of a ring bind RuBisCo while the other three are in the GroES-accepting conformation, suggesting a mechanism for how GroEL can recruit GroES without releasing its bound substrate [36]. Regardless of the precise mechanism, stretching is believed to prime the substrate for efficient folding during the following encapsulation step. Secondly, steric confinement within the central cavity accelerates the folding rate by constraining the conformational freedom of the substrate. This restriction minimizes the formation of kinetically trapped intermediates that could otherwise impede the folding process. For instance, encapsulation of DapA within the folding cavity enhances the folding rate of this protein by more than 30-fold by promoting segmental structure formation [34]. Finally, the charged character of the cavity's surface restricts the mobility of the encapsulated substrate, while hydrophobic interactions between the

substrate and the disordered C-terminal residues coming from the bottom of the ring cavity of GroEL contribute to substrate remodeling [17,35].

Tuning the GroEL–GroES nanomachine using a molecular plug-in

A new layer was recently added to the complexity of the GroEL–GroES nanomachine as a result of our work on CnoX. CnoX , which is found in representatives of the Proteobacteria, Bacteroides, Cyanobacteria, and many other phyla, displays an N-terminal thioredoxin-like domain fused to a C-terminal tetratricopeptide (TPR) domain (Figure 2). While thioredoxin domains are typically found in oxidoreductases [37], TPR domains often mediate protein–protein interactions [38]. Initial investigations on CnoX showed that this protein was part of the protein homeostasis network, functioning as a chaperone preventing its substrates from aggregation [39,40]. In *E. coli*, but not in *Caulobacter crescentus*, chaperone activity is increased by the reversible N-chlorination of several basic residues in the TPR domain, which augments the affinity of this region for unfolded polypeptides [39,40]. Interestingly, we found that CnoX is more than a chaperone; it also provides, via its thioredoxin domain, redox protection to its substrates, preventing their cysteine residues from irreversible oxidation.

Figure 2



The chaperedoxin CnoX forms a functional complex with GroEL. **(a)** Structure of *E. coli* CnoX (PDB: 3QOU). The N-terminal thioredoxin domain, with its two cysteine residues, is shown in green. The C-terminal TPR domain is shown in pink. The cysteine residues present in the thioredoxin domain are indicated. **(b)** Side view of the structure of the GroEL–CnoX complex (PDB: 7YWY). GroEL is shown as a solvent-accessible surface and CnoX as cartoon. The equatorial, intermediate, and apical domains of GroEL are shown in slate, orange, and light cyan, respectively, and CnoX is shown in pink. **(c)** CnoX, which forms a stable complex with GroEL via its C-terminal α-helix, interacts with incoming substrates for GroEL. If the substrate presents oxidized cysteine residues (to a sulfenic acid or in a disulfide bond), CnoX reacts with the substrate via the cysteines of its thioredoxin domain, and a mixed disulfide is formed. The cytoplasmic reducing pathways reduce the mixed disulfide, releasing the substrate in a reduced, folding-competent state. PDB; Protein Data Bank.

CnoX protects its substrates from aggregation but is unable to help them (re)-gain their native conformation. To that purpose, CnoX uniquely cooperates with the GroEL–GroES system for active refolding [39,40]. Intrigued by this property of CnoX, we sought to investigate the molecular details of the functional relationship between CnoX and GroEL–GroES [41]. We found that CnoX forms a stable complex with GroEL in an ATP-independent manner (Figure 2). Binding of GroES to GroEL induces CnoX release [41]. Cryoelectron microscopy provided crucial structural information on the GroEL–CnoX complex, showing that the highly conserved C-terminal α-helix of the TPR domain of CnoX binds the apical domain of GroEL, outside the substrate-binding site [41]. Furthermore, we identified complexes in which CnoX, bound to GroEL, forms mixed-disulfide bonds with GroEL substrates, including several obligate clients. We proposed a model in which the thioredoxin domain of CnoX reacts with GroEL substrates that present oxidized cysteine residues (to a sulfenic acid or in a disulfide bond) that would otherwise impede folding, resulting in the formation of a CnoX-substrate mixed disulfide [41]. The reducing pathways active in the cytoplasm would then reduce the mixed disulfide, releasing the substrate in a reduced, folding-competent state. Finally, the binding of GroES to GroEL occludes the CnoX-binding site on the chaperonin, triggering CnoX release from GroEL and encapsulation of the substrate within the folding cage for folding. Thus, in our model, CnoX functions as a molecular plugin that provides redox quality control for GroEL substrates (Figure 2). Proteins exhibiting structural features similar to CnoX have been identified in eukaryotes [41], which suggests that molecular plugins functioning with Hsp60 proteins may have been conserved through evolution.

Conclusions

In conclusion, despite several decades of research that have delivered a wealth of information on the GroEL–GroES system, additional layers of complexity have been uncovered in recent years, raising new questions and perspectives. The application of advanced techniques such as cryoelectron microscopy promises to elucidate the high-resolution structures of GroEL in complexes with a variety of substrates, including those engaged in mixed-disulfide bonds with CnoX. This approach will further enhance our understanding of the intricate molecular interactions and conformational dynamics integral to the protein folding process. Furthermore, the identification of CnoX as a critical molecular plugin for GroEL–GroES opens a new avenue of exploration; investigating the existence and roles of similar chaperonin plugins in bacteria and eukaryotes could unveil previously unrecognized aspects of protein quality control and redox regulation. Investigating the interplay between redox states and protein folding, particularly in the context of CnoX and GroEL–GroES interactions, could unravel how redox alterations influence chaperonin functionality and substrate folding, offering fresh insights into cellular redox homeostasis. Finally, probing the evolutionary conservation and divergence of chaperonin systems in emerging bacterial models, such as bacteria from the microbiota, and the role of molecular plugins like CnoX could provide valuable perspectives on how protein folding mechanisms have evolved to adapt to different environments. Collectively, future research on the GroEL–GroES nanomachine will not only deepen our understanding of molecular chaperones but could also advance biotechnological and medical applications by opening the way for the enhanced production of therapeutic proteins in bacteria or the design of novel therapeutic compounds like antibiotics.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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