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REVIEW

Protein Folding in the Cell: On the Mechanisms of Its Acceleration

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Abstract—The mechanisms responsible for protein folding in the cell can be divided in two groups. The ones in the first group would be those preventing the aggregation of unfolded polypeptide chains or of incompletely folded proteins, as well as the mechanisms which provide for the energy-consuming unfolding of incorrectly folded structures, giving them a chance to begin a new folding cycle. Mechanisms of this type do not affect the rate of folding (it occurs spontaneously), yet considerably increase the efficiency of the entire process. By contrast, the mechanisms belonging to second group actually accelerate protein folding by exerting a direct influence on the rate-limiting steps of the overall reaction. Although not a conventional one, such a classification helps define the topic of this review. Its main purpose is to discuss the ability of chaperonins (and that of some chaperones) to interact directly with substrate proteins in the course of their folding and thus accelerate the rate-limiting steps of that process. (Mechanisms of protein folding acceleration produced by the action of enzymes, e.g., peptidyl-prolyl *cis/trans* isomerase and protein disulfide isomerase, are not considered in this review.) Specific cases demonstrating an accelerated folding of some proteins encapsulated in the bacterial chaperonin GroEL cavity are considered, and the conditions favoring such acceleration are examined. Experimental data supporting the notion that the structure and functional properties of GroEL are not optimal for an effective folding of many of its substrate proteins is discussed. The current status of research on the mechanism behind the active participation of different subunits of eucaryotic cytosol chaperonin (CCT) in the final steps of the folding of actin and tubulin is reviewed. Particular attention is devoted to steric chaperones, which dramatically accelerate the formation of the native structure of their substrate proteins by stabilizing certain folding intermediates. The structural foundations underlying the effect of the subtilisin pro-domain on the folding of the mature enzyme are considered. The prospects of future studies into the mechanisms responsible for accelerating protein folding in the cell are commented upon.

Key words: protein folding, rate-limiting steps, chaperonins, GroEL, CCT, domains, actin, tubulin, steric chaperones, pro-domains, subtilisin

To become functionally active, a newly synthesized polypeptide chain must fold into a unique three-dimensional structure. Understanding how this is achieved is a fundamental biological problem. Although experiments *in vitro* have firmly established that the information on the three-dimensional structure of a protein is contained in its amino acid sequence [1, 2], protein folding in the cell cannot be regarded as occurring spontaneously. Since the late 1980s, it has become clear that many proteins require assistance to fold in the cell and that this is provided by helper proteins, collectively known as molecular chaperones [3-7]. A special group of helper proteins, the so-called foldases (peptidyl-prolyl *cis/trans* isomerase [8, 9] and protein disulfide isomerase [10, 11]), assist protein folding by catalyzing the rate-limiting isomerization reactions.

The chaperones involved in the folding of nascent polypeptide chains perform repeated cycles of binding and release of substrate proteins, regulated by ATPase activity typical of these helper proteins, and by cofactor proteins. The action of chaperones is based on two different mechanisms. The first mechanism, used by Hsp70, consists of maintaining the polypeptide chain in a state capable of productive folding, which occurs spontaneously after the release of unfolded chains into solution. Thus, interaction with the chaperone has no effect on the folding process *per se*.

The second mechanism is used by chaperonins, whose large cylindrical complexes create physically isolated compartments destined for the folding of polypeptides, partially folded or misfolded proteins, which become encapsulated inside the central cavity. Since only

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one molecule of the substrate protein can fit inside the cavity, its folding takes place under conditions, which completely exclude aggregation. However, the role of chaperonins clearly is not limited to their ability to create isolated compartments where proteins can fold spontaneously under conditions simulating infinite dilution (the "Anfinsen cage" model) [12]. A large body of information accumulated to date supports the notion that chaperonins can play an active role in protein folding, accelerating this process and considerably increasing its efficiency.

Thus, the bacterial chaperonin GroEL, in cooperation with its co-chaperonin GroES and using the energy of ATP binding, has been shown to effectuate "forced unfolding" of a substrate protein trapped in a misfolded condition, allowing it a new opportunity to fold. This process can be repeated until all protein molecules achieve the native state [13-16]. The "iterative annealing" model, describing such a mechanism, leaves room for a suggestion that parallel to the increase in the efficiency of the folding process, some acceleration of the folding may also take place (one possibility is that, having been unfolded and ejected from the cavity, the protein may undergo fast folding in free solution). Another mechanism of folding acceleration can be based on interaction between the walls of the inner chaperonin cavity and the encapsulated protein during its folding. A distinctive feature of such a mechanism would be the possibility of the chaperonin participating directly in the folding process.

Which one of these two mechanisms (though they are not mutually exclusive) offers a better explanation for the effects observed experimentally? This has been the subject of some lively debate in recent years, which led to a number of special studies performed on the chaperonin GroEL. One aim of the present review is to consider the results of these investigations and to discuss them in the light of currently available information. Another topic of the paper is the mechanism responsible for the active participation of CCT, the chaperonin of eucaryotic cytosol, in the folding of actin and tubulin. In conclusion, we will turn to a particular group of chaperones whose main function is to accelerate the folding of their substrate proteins by delivering steric information at the final steps of the process (steric chaperones).

BACTERIAL CHAPERONIN GroEL: A GENERAL-PURPOSE MACHINE OF MODERATE PROFICIENCY

The *Escherichia coli* (*E. coli*) chaperonin system GroEL–GroES plays an important biological role, essential for the folding of a number of cytosolic proteins [17-19]. GroEL is composed of 14 identical 58 kD subunits, organized in two heptameric rings (toroids) which form a cylindrical structure with two separate inner cavities [20-22]. The apical domains of the subunits expose hydrophobic binding surfaces toward the ring center and engage in multiple contacts with a non-native substrate [23, 24]. In each subunit, the apical domain is connected via a hinge-like intermediate domain with an equatorial ATPase domain that mediates most intersubunit contacts within and between GroEL rings. GroES, which is a cofactor of GroEL, is built of seven 10 kD subunits, forming a dome-shaped heptameric ring that caps the ends of the GroEL cylinder [21, 25, 26].

The functional cycle of the GroEL–GroES system can be described as follows (Fig. 1, I). A newly synthesized polypeptide chain or a partially folded protein binds with highest affinity to GroEL in its nucleotide-free state (stage 1). The binding takes place in the *cis* ring, by way of hydrophobic interactions between the apical domains and the hydrophobic regions of the substrate protein. The apical domains of several (at least three) subunits are involved in the interaction [24]. The subsequent binding of seven ATP molecules to the equatorial domains of the *cis* ring subunits (stage 2), which is positively cooperative, inhibits the binding of ATP with the *trans* ring [27, 28].

This creates an asymmetry between the rings, leading to their alternating functioning [29-32]. A recent crystallographic study of the complex formed by GroEL with KMgATP showed that the binding of the nucleotide is accompanied by previously unnoticed domain rotations and a 102° rotation of the apical domain surface helix 1. This results in a large lateral displacement of, and a dramatic reduction of hydrophobicity in, the apical domain surface [33].

The binding of ATP initiates the most important step in the chaperonin cycle, namely, the formation of a closed cavity with the participation of GroES (stage 3). The apical domains swing upwards by 60° relative to the equator and twist around the "long axis" of the domain by about 90°, resulting in an interaction with the "mobile loop" of GroES [21, 22]. The domain movements dramatically reshape the cavity, doubling the volume of the cavity and changing the polypeptide binding properties of the cavity lining, which becomes largely hydrophilic. This switch in the character of the cavity lining triggers the dissociation of the non-native polypeptide from apical domains and their encapsulation inside the cavity. If the protein molecular mass does not exceed 60 kD [34], it becomes encapsulated and free to begin folding in the *cis* ring cavity. For clarity, the binding of ATP and GroES are shown in the figure as two distinct steps; in fact, both processes occur simultaneously.

The encapsulation time is limited by the rate of ATP hydrolysis in the equatorial domains of the *cis* ring (on average, 10-15 sec at 25°C) (stage 4) [29, 35]. ATP hydrolysis weakens the interaction between GroEL and GroES, priming the system for GroES and substrate release. Upon completion of hydrolysis (state *e* in Fig. 1, I), a signal is transmitted to the equatorial domains of the *trans* ring, increasing their affinity to ATP and causing

seven ATP molecules to bind to that ring. The binding of ATP to the *trans* ring sends an allosteric signal to the *cis* ring. This, along with the concurrent binding of GroES to the same *trans* ring, induces the release of GroES, ADP, and the substrate protein from the *cis* ring (stage 6) [27, 36-39].

The figure also shows that at stage 5 a new molecule of unfolded protein binds to the *trans* ring to start its folding cycle. This takes place alternatively in the *cis* and *trans* rings and is regulated by allosteric signals, which are transmitted via interdomain and intersubunit contacts. During this process the energy of ATP binding is used to unfold the substrate protein, and the energy of ATP hydrolysis, to achieve the dissociation of ADP, GroES, and of the folding product, which, in the case shown in Fig. 1, is the protein in its native conformation.

The mechanism described in Fig. 1, I rests on the assumption that the role of the chaperonin is limited to creating a sequestered microenvironment where folding to the native state can proceed while the substrate protein is protected from aggregation (the "Anfinsen cage" model) [12]. Although this may sometimes be the case, numerous experiments have shown that the folding of a certain number of proteins requires multiple cycles of GroEL functioning, with the substrate protein being ejected from the cavity at each round of ATP hydrolysis, whether it has reached the native state or not [40-42]. This pointed to the ability of the chaperonin to unfold a protein, which has become trapped in a misfolded condition and is incapable of reaching its native state [15, 43-47].

To describe such a mechanism, the "iterative annealing" model has been proposed [48, 49], based on the view

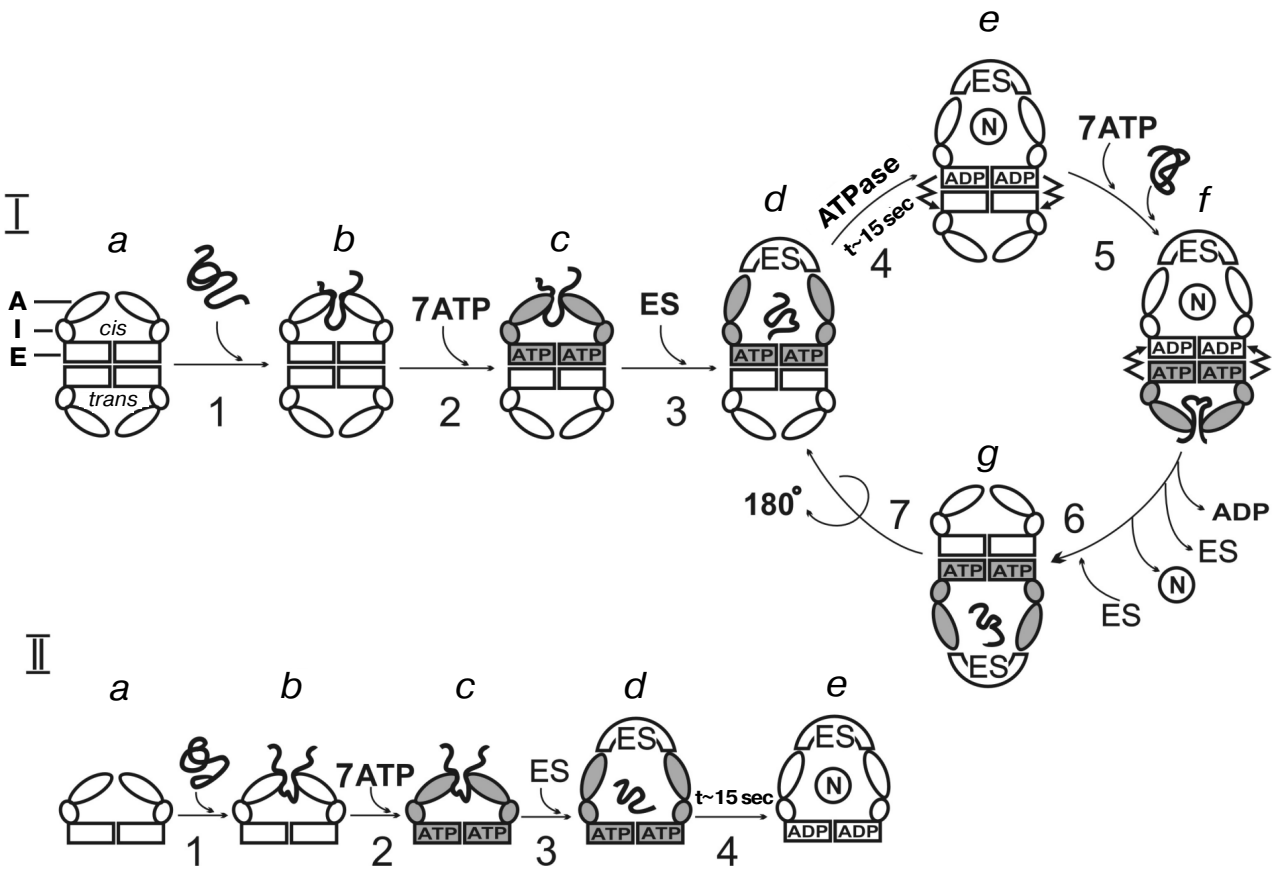

Fig. 1. Functional cycle of GroEL–GroES system. I, native (a two-ring) chaperonin; II, a mutant containing one ring; 1) binding of an unfolded protein; 2) the binding of ATP inducing considerable conformational changes in the *cis* ring (shaded in gray); 3) binding of GroES, which occurs simultaneously with the ATP binding; 4) ATP hydrolysis. Stages 1-4 take place in the *cis* ring of the native chaperonin or in the single ring of the mutant. In the second case, the cycle is terminated by the formation of a native protein, which remains encapsulated in the closed cavity (state *e*). In the two-ring chaperonin, completion of ATP hydrolysis is accompanied by the transmission of an allosteric signal (shown by jagged arrows) to the equatorial domains of the *trans* ring. The result is a marked increase in their affinity to ATP. A new unfolded protein molecule binds to the *trans* ring (stage 5). The binding of ATP to the *trans* ring sends a signal to the equatorial domains of the *cis* ring. Together with the effect produced by the simultaneous binding of GroES to the *trans* ring (stage 6), this causes the opening of the cavity, the dissociation of ADP and GroES from the *cis* ring, and the release of the protein that has been folded into native conformation (stage 6). Structure *g* is formed, which is similar to structure *d* rotated by 180°. Abbreviations: A, apical domain; I, intermediate domain; E, equatorial domain; ES, GroES; N, native protein conformation. Taken from [39] with alterations.

that the rate-limiting step in slow protein folding can be the intramolecular reorganization of misfolded and trapped protein segments, which requires the protein to unfold to a certain degree [15]. According to this model, GroEL uses the energy of ATP binding and hydrolysis to unfold misfolded substrate molecules and release them either into the protected central cavity or to the exterior, so that the misfolding is relieved and forward folding can continue. Once again, the incompletely folded protein undergoes further iterations until it achieves the native state.

Compelling experimental evidence for the existence of such a mechanism could be obtained by demonstrating directly that GroEL is indeed able to "force" the unfolding of misfolded proteins, using the energy of ATP binding and hydrolysis in the course of repeated functional cycles. Such evidence was obtained in the study by Shtilerman et al. [16] carried out on ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO), whose folding is stringently dependent on GroEL–GroES–ATP both *in vivo* and *in vitro* [50, 51]. The unfolding of RuBisCO was probed through a hydrogen-tritium exchange approach. In the absence of the chaperonin system, RuBisCO folding was blocked due to the formation of a kinetically trapped intermediate with a core of 12 amide hydrogens that were strongly protected from exchange with bulk solvent.

This folding intermediate could be captured efficiently by GroEL, but such binding did not cause it any observable destabilization. However, when both GroES and ATP were added to the GroEL–RuBisCO binary complex, a rapid exchange of nearly all (~10) of the protected hydrogens took place, suggesting that the nonnative RuBisCO had been largely or totally unfolded. The unfolding was complete in 13 sec, i.e., within a single round of GroES and ATP binding. A non-hydrolyzable ATP analog was as effective as ATP, indicating that the energy for substrate protein unfolding is derived from the binding rather than the hydrolysis of nucleoside triphosphate.

These results are in good agreement with the structural data we discussed above while describing the functional cycle of GroEL. Upon initial binding to GroEL (state *b* in Fig. 1, I), the substrate does not undergo any significant unfolding. This binding can be regarded as "passive", similar to the binding of substrate proteins with the chaperone Hsp70 [52]. The subsequent binding of ATP and GroES (states *c* and *d*) induces global structural changes in GroEL: the apical domains twist upward and outward, causing the substrate binding sites, located on different subunits of the heptameric ring, to move away from each other [21, 22]. Such displacement can reach 20 Å and result in a noticeable "stretching" of the substrate protein structure, and in its partial unfolding. This effect, powered by the energy of ATP binding, has been called "forced unfolding" [16].

Experiments with substoichiometric GroEL concentrations, conducted at a GroEL/RuBisCO ratio of 1 : 20, further showed that complete folding involves multiple cycles in which the substrate protein is bound and released repeatedly until it reaches its native state [16], supporting the idea that apart from the energy of ATP binding, the energy of ATP hydrolysis is also required to support the repeated cycles. It was presumed that the folding can take place both in the chaperonin cavity and in free solution.

The study by Shtileman et al., which solidly corroborated the concept of "forced unfolding", has nonetheless left several important problems unresolved, particularly the question of whether the "forced unfolding" influences the rate of protein folding in the cell [53]. Another problem has to do with the existence of mechanisms able to accelerate protein folding in the isolated chaperonin cavity.

A remarkable advance in this field was made in a recent work by Brinker et al. [54], which demonstrated an accelerating effect of the chaperonin on the folding rate. The conditions for an efficient renaturation of RuBisCO *in vitro* were elaborated, and a comparative study was performed. The results showed that the folding of RuBisCO in the closed GroEL–GroES cavity was four times faster than spontaneous folding, suggesting a catalytic role of the chaperonin.

To understand the mechanism behind this effect, it was necessary to determine whether the iterative annealing phenomenon makes any contribution to the rate acceleration. With this aim, a GroEL mutant was used, containing just one heptameric ring instead of the two rings characteristic of the native chaperonin (Fig. 1, II) [30, 55, 56]. Such a mutant, called SR1, is capable of binding GroES, but cannot release it, owing to the absence of an allosteric signal from the *trans* ring (compare I and II in Fig. 1), and, consequently, is unable to perform multiple cycles of functioning. A comparative study of RuBisCO folding rates in the GroEL–GroES system, where the iterative annealing mechanism functions, and in the SR1–GroES system, where it does not, has shown these rates to be similar in both cases. This indicates that the rate acceleration in the cavity of the chaperonin is unrelated to its repeated cycles of functioning.

The fact that similar experiments carried out with another substrate protein, rhodanese, whose molecular mass (~33 kD) is considerably lower than that of a RuBisCO subunit (~50 kD), revealed no accelerating effect of GroEL, suggests that this effect may depend on the size of the substrate protein molecule relative to the dimensions of the central cavity. The authors propose that, in the environment of the chaperonin cage, formation of certain kinetically trapped intermediates is either avoided or their progression toward the native state is facilitated.

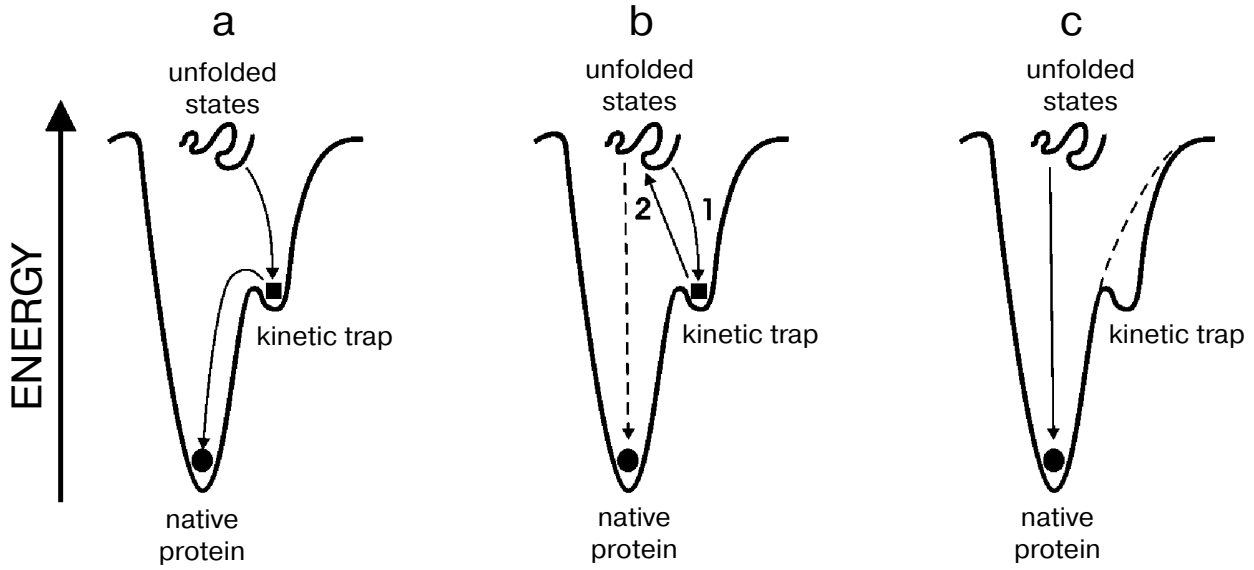

Fig. 2. Schematic representation of the mechanisms underlying GroEL effect on protein folding using simple energy diagrams. a) Spontaneous folding in a closed chaperonin cavity. If an intermediate gets caught in a kinetic trap, folding will slow down. b) A model of folding acceleration as a result of "iterative annealing". An intermediate caught in a kinetic trap inside the chaperonin cavity or in solution (arrow 1) is unfolded upon interaction with the GroEL–ATP–GroES system (arrow 2), and gains the capacity to begin a new cycle of folding. c) Folding acceleration stemming from confinement of the substrate protein in a narrow hydrophilic chaperonin cavity, where the formation of trapped intermediates can be avoided. This results in a smoother energy landscape (the dotted line). Taken from [56] with alterations.

The above study thus offers a new perspective of how chaperonin can influence protein folding in the cell, suggesting a direct effect of the chaperonin cavity walls on the protein during the folding process. Depending on the structure of the substrate, different effects, schematically represented in Fig. 2, may occur. The figure shows cross sections of the funnels representing the energy landscapes of the protein folding reaction. The width of the funnels, that represents the entropy, becomes steadily narrower for conformations closer to the native state. The walls of the funnels are not completely smooth, but display local energy minima like the one shown in the figure, which can be regarded as kinetic traps [57].

Case (a) is described by the "Anfinsen cage" model, where the role of the chaperonin is restricted to creating conditions, which prevent aggregation. A protein folds spontaneously; the formation of a kinetically trapped intermediate slows down the folding, but the chaperonin takes no part in the unfolding of this intermediate. Such a scenario was observed in the experiments performed by Brinker et al. [54] on rhodanese.

Case (b) corresponds to the model where protein folding is accelerated by iterative annealing. This mechanism failed to explain the rate acceleration data obtained by Brinker et al. with RuBisCO [54]. The authors suggested a new mechanism, shown in Fig. 2c. According to it, if the size of the folding substrate molecule fits the dimensions (and possibly the architecture) of the chaperonin cavity, it will become stabilized in a more compact conformation which is not likely to produce an intermediate that would fall into a kinetic trap. This flattens the energy landscape and expedites the reaction.

It follows from the results discussed above that a distinction between cases (b) and (c) can be made by studying the folding process under conditions where repeated cycles of protein binding and release are not possible. In the work of Brinker et al. [54], it was achieved by using a single-ring GroEL mutant in complex with GroES. Another approach can be based on the use of a native two-ring GroEL in the absence of GroES. Under these conditions, the folding would occur in an open cavity and could be studied as such.

Such an approach has been employed by Persson et al. who examined the kinetics of carbonic anhydrase folding in the presence and absence of GroEL [58]. The nearly two-fold lowering of the activation energy in the presence of GroEL led the authors to suggest that contact with the chaperonin leads to a folding route with a flatter energy landscape than that of spontaneous reaction. These results, together with the data of Brinker et al. [54], pointed to the capacity of the chaperonin cavity walls to influence the folding process directly, but left open the question as to the mechanism by which that influence occurs.

A step forward in this direction was made by Coyle at al., who chose lysozyme as the substrate protein [59]. Since the mechanism of spontaneous folding of this

enzyme has been extensively studied [60], the authors were in a good position to examine the different steps of the folding process in the absence and presence of GroEL. The native structure of lysozyme is stabilized by four disulfide bonds and consists of two domains (α and β), with the active center in between. It was established by *in vitro* experiments that both domains are relatively autonomous structural units, capable of folding independently of one another [61, 62], the final step of folding into the native conformation being the slow reorganization that results in the establishment of interdomain interactions [63].

While the folding of disulfide-intact lysozyme is unaffected by the full GroEL–GroES–ATP machinery, GroEL alone binds to non-native lysozyme and modestly but significantly accelerates the rate of formation of native protein. For this reason, lysozyme can serve a model for the effect of GroEL on some proteins that transit rapidly through chaperonins *in vivo* and therefore are likely to avoid encapsulation under GroES [53, 64].

An investigation of spontaneous lysozyme folding at neutral pH has shown that it involves the sequential formation of three distinct folding intermediates. Initially, there is a rapid hydrophobic collapse ($\tau < 2$ msec), followed by the second fast phase ($\tau = 25$ msec), when α-domain is formed. The third, slow phase ($\tau = 625$ msec) involves the formation of the β-domain and a reorganization of non-native tertiary interactions leading to the formation of near-native species. The rate-limiting step in the final phase is mainly the docking of preformed α- and β-domains to generate the complete native structure.

In the presence of GroEL, a marked increase in the rate of the slow phase was detected ($\tau = 420$ msec) as compared to spontaneous folding. This acceleration requires the binding of lysozyme within the hydrophobic central cavity, since presaturation of the cavity with denatured mitochondrial malate dehydrogenase blocks the ability of GroEL to enhance folding. To explain the mechanism of the acceleration, several possibilities have been considered and tested experimentally. The authors managed to map the pathway of lysozyme folding in the presence of GroEL and to demonstrate that the chaperonin does not create a distinct folding pathway, nor does it affect the fast folding phase. Rather, GroEL assists folding by facilitating rate-limiting domain docking events in the final slow phase.

Possibly, the role of the chaperonin is determined by its ability to establish temporary contacts with intermediates of the folding reaction, promoting domain rearrangements and the emergence of correct interactions between them [65, 66]. To do so, minor specific adjustments that do not entail global unfolding or significant domain destabilization could suffice. One way of achieving this could be the rupture of hydrophobic interactions stabilizing an incorrect structure of the intermediate; a folding route with a flatter energy landscape than that of the spontaneous reaction would be created as a result [21, 67].

The experiments with lysozyme as a model substrate, whose entry into the central chaperonin cavity is associated neither with the binding of GroES, nor with the use of ATP binding energy, have shed some light on the problem of how GroEL selects its substrates *in vivo* [53]. It is known that there are far more newly made polypeptides than GroEL–polypeptide complexes in a cell [12, 68]. Therefore, despite the ability of GroEL to efficiently bind most unfolded proteins, there must exist a mechanism ensuring its preferential interaction with the roughly 10% of *E. coli* proteins that actually require the GroEL–GroES–ATP machinery to fold.

Such selectivity could be achieved if smaller proteins like lysozyme, whose folding does not require GroEL, were able to bind rapidly in an open cavity, undergo the process of folding, and dissociate from GroEL surface before the binding of GroES initiates the full reaction cycle. A competition between such smaller proteins and "true" substrates would be unlikely given the time lag between the binding of a "true" substrate and that of GroES, during which time fast-folding species would have the opportunity to complete folding and leave. It is known that this delay could be as long as 30 sec [53].

However, more stringent control is present in the case of a particular group of "true" substrate proteins. Thus, the binding of denatured malate dehydrogenase or RuBisCO to GroEL accelerates the rate of GroES and nucleotide binding by a factor of 50 [69], i.e., the binding of these substrates would appear to catalyze their encapsulation, eliminating the delay period that is needed to allow folding on the GroEL surface. In this manner, the "true" substrates, whose folding absolutely requires the participation of GroEL and its co-chaperonin, are given the "green light".

Turning back to the work of Brinker et al. [54], one should note that along with an in-depth analysis of the mechanisms underlying the GroEL effect on the rate of protein folding, that study contains information, which is relevant to issues of a different nature. It attracts attention to the unusually broad substrate specificity for which GroEL is well known, its substrates ranging from RuBisCO, whose structure is well suited for the chaperonin to display its accelerating effect, to rhodanese, that cannot be influenced in this way at all.

GroEL does appear to be rather promiscuous in choosing substrates capable of binding with its apical domains. As mentioned above, the main condition is the presence of surface-exposed hydrophobic patches that readily bind to the hydrophobic sites of the apical GroEL domains. A question arises as to the biological purpose of such substrate promiscuity, which keeps GroEL from attaining maximum efficiency as a "folding machine".

An attempt to answer this question was made in a recent study by Weissman and collaborators [70]. One objective of this work was to find out whether the GroEL–GroES structure can be optimized in such a way

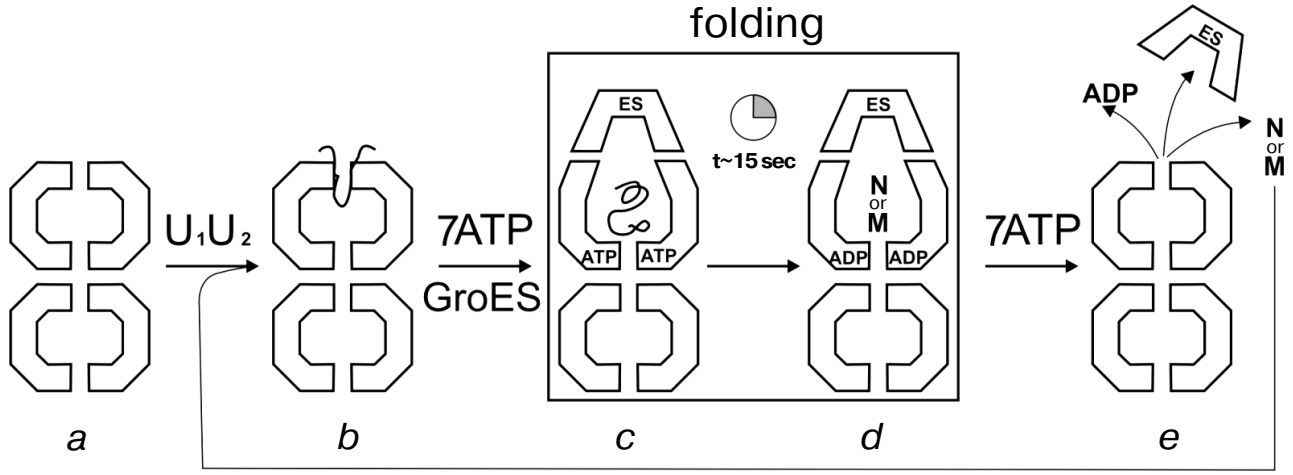

Fig. 3. Folding cycles of proteins capable (U_1) and incapable (U_2) of folding into native conformation inside the GroEL–GroES cavity within 13-15 sec (25°C). GroEL (state *a*) can bind unfolded U_1 and U_2 states, as well as misfolded or incompletely folded U_2 states (M). N, native state; ES, GroES. A detailed description of the GroEL–GroES cycle is presented in Fig. 1. Taken from [71] with alterations.

as to make it capable of folding predominantly a particular substrate. Another was to investigate the effect of such optimization on the properties of the GroEL–GroES system as a general-purpose "folding machine".

The authors prepared GroEL and GroES mutants that were most efficient at folding a single substrate. The model substrate used in the selection procedure was the green fluorescent protein (GFP). An optimal result (6-8-fold increase in the yield of folded protein compared to the wild-type chaperonin) was achieved with mutants containing mutations in both GroEL and GroES, which indicated complex functional interplay between the two partners. In the case of GroEL, the mutations were located either near the ATP-binding site or in the intermediate domain. Generally, these mutations slowed down ATP hydrolysis when GroES was present in the *cis* ring of the active folding complex. As a result, the time a substrate protein would spend sequestered inside the inner cavity was increased considerably, up to 50 instead of 15 sec characteristic of the wild type GroEL–GroES system. The positive effect these mutations had on the efficiency of GFP folding shows that the time allotted for folding in a wild-type GroEL–GroES cavity is probably too short to ensure a successful folding of the model substrate.

Of the mutations introduced in GroES, the largest effect was brought about by the change of Tyr71 to either a polar or a charged hydrophilic residue. In the structure of the GroEL–GroES complex, Tyr71 contributes to a large hydrophobic patch on the ceiling of the folding chamber. Therefore, changing it to a more hydrophilic residue dramatically increases the hydrophilic nature of the folding cavity. In their discussion of the above results, Bukau and coauthors [71] speculate that the hydrophilic replacement at Tyr71 helps the *cis*-folding of GFP by facilitating the detachment of GFP from GroEL–GroES and/or by "smoothing" the energy landscape as previously proposed for RuBisCO [54].

The results obtained in this study called attention to the structure of the folding chamber ceiling and thus offered a new approach to examining the mechanism of active participation of the GroEL–GroES system in protein folding. An essential part of this approach is an optimization of the folding conditions, which ensures favorable interactions of the folding intermediate with the walls of the inner cavity.

However, the optimization of GroEL–GroES structure for the folding of GFP had far-reaching consequences: it endangered the cell's survival, since the mutants became unable to fold a variety of natural substrates. The study thus revealed a conflict between specialization and generalization of chaperonins, as increased GFP folding comes at the expense of the ability of the GroEL–GroES system to fold other proteins. Since the multitude of GroEL substrates will have a range of different folding rates, the authors [70] hypothesized that the wild-type ATPase rate could be an evolutionary compromise designed to accommodate the folding of a number of substrates. It seems reasonable to suggest that a short folding time, which may be close to optimal only for a limited set of substrates, is nevertheless acceptable for many others, whose folding can be achieved after several rounds of GroEL binding. Figuratively speaking, GroEL–GroES appears to be "a folding machine for many but master of none; it is not a finely tuned instrument, but rather it is a robust and sturdy machine to work well enough on a wide range of substrates necessary to ensure cell survival" [71].

The aforesaid is illustrated by Fig. 3, which schematically shows the functional cycles of the GroEL–GroES

system as it assists the folding of two types of substrate proteins: those that are capable of folding into the native conformation within an encapsulation period not exceeding 15 sec, and those that are not. In the first case, a single cycle is sufficient for complete folding, with the energy of ATP binding used to expel the unfolded substrate into the inner chaperonin cavity, and the energy of ATP hydrolysis to release the native protein. In the second case, the first cycle produces a protein that has not had the time to fold and needs to bind to GroEL once again to repeat the folding cycle.

What are the factors determining the period of encapsulation that is sufficient for the folding of a protein in the GroEL–GroES cavity? The experimental data pointing to a possibility of the cavity walls directly participating in the process of folding suggest that this effect could play a role. One can speculate that the proteins whose size and shape fit closely the dimensions and architecture of the cavity would fold faster. It seems likely that further studies in this direction will provide useful insights into the mechanism of GroEL functioning.

EUCARYOTIC CYTOSOLIC CHAPERONIN: A COMPLEX HETEROOLIGOMERIC STRUCTURE AS AN INSTRUMENT FOR DIFFICULT FOLDING TASKS

Unlike GroEL, which will bind to any protein presenting stretches of hydrophobic residues, the cytosolic chaperonin of eucaryotic cells (CCT) recognizes specific binding determinants in its substrates. The main substrates of this chaperonin are actin and tubulin, although some other proteins too are now being mentioned as potential CCT substrates [72, 73]. Together with chaperonins from archaebacteria (thermosomes), CCT belongs to group II chaperonins that are distinguished from group I chaperonins (including GroEL) by a number of properties.

The most important of these properties is the heterooligomeric structure. Each of the two rings of CCT consists of eight different subunits positioned in a precise arrangement. These subunits recognize specific epitopes present in actin and tubulin, and interact with them in a particular manner. Hence the high substrate selectivity of this chaperonin and its ability to stabilize both cytoskeletal proteins in open and quasi-folded conformations. This task is too complex for GroEL: neither actin, nor tubulin can be folded by this chaperonin, even though the size of their domains (~56 kD) is optimal for encapsulation in the GroEL cavity [34, 71]. Although GroEL can bind and release actin and tubulin in an ATP-dependent manner, it cannot promote their folding, which is why these proteins cannot be produced in native forms by bacteria [74].

The second distinctive feature of group II chaperonins is the absence of a partner co-chaperonin. The three-dimensional structure of the thermosome, which is very similar to other group II chaperonins, reveals the same three-domain arrangement originally described in the GroEL monomer, with a conserved equatorial domain but a less similar apical domain responsible for the binding of substrates [75-78]. The apical domain of the thermosome [76], like that of CCT [79], has an extra region located at the tip of the domain, which is not present in group I chaperonins. This helical region, protruding toward what would be the center of the cavity in an intact chaperonin, has been proposed to act as a built-in lid, capable of closing the cavity in a particular functional state of the chaperonin, a role equivalent to that of co-chaperonin GroES.

Based on a combination of X-ray crystallography data, cryoelectron microscopy, and biochemical and biophysical studies carried out on CCT in its different conformational states (in the absence of ligands, in complexes with actin and tubulin without nucleotide, and in the presence of a non-hydrolyzable ATP analog) [80-85], one can suggest a hypothetical reaction cycle for this chaperonin (Fig. 4). Only the functioning of the *cis* ring is shown; inter-ring communications, as yet scantily known, are not considered.

At the first stage, the binding of substrate protein to hydrophobic regions of the exposed apical domains takes place. As opposed to GroEL, this binding is specific. Only a few of the eight subunits, each of which is encoded by a unique gene [87], recognize particular epitopes at the surface of actin or tubulin [83, 88-91]. In the case of actin, it can be the δ and ε subunits or the δ and β subunits (details are given in Fig. 5). It has been shown that polar and electrostatic interactions are involved in the binding of tubulin [79].

CCT seems to recognize actin and tubulin in their quasi-folded state, with domains already formed [81, 92]. It binds the two domains of the two cytoskeletal proteins using opposite regions of the CCT ring. In this process, the N- and C-terminal domains move apart, and an extended (open) conformation is stabilized. This is schematically shown in Fig. 4, state *a*. The binding of ATP induces a 70° clockwise rotation of the apical core domains, which orients the helical protrusions towards the center of the cavity. The chaperonin cavity is then completely sealed off as a result of the apical domains tilting 30° downward. It should be noted however that according to some recent data, complete closure of the cavity only occurs following ATP hydrolysis [85].

Unlike in the case of GroEL, encapsulation of the substrate is not accompanied by its release into the cavity; rather, as shown in Fig. 4*b*, it remains bound to the chaperonin. The energy of ATP binding (and possibly hydrolysis) is then used to force the folding of the substrate, whereby the open conformations of actin and tubulin evolve toward compact, quasi-native (or native) structures that are still bound to the chaperonin and not

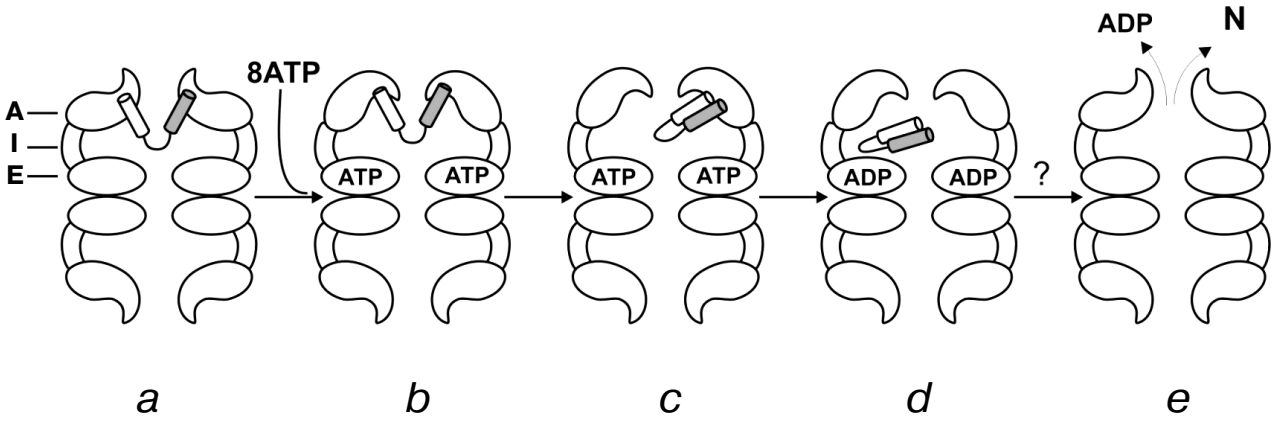

Fig. 4. Functional cycle of the chaperonin CCT. The apical domains of specific subunits bind actin and tubulin in their quasi-folded state, with domains already formed (shown as two cylinders). In the absence of ATP, the helical protrusions of the apical domains, which take part in the binding, are turned outward from the cavity and expose their hydrophobic regions (state *a*). The binding of ATP (according to some data, also ATP hydrolysis, see text) induces a movement of the apical domains, whose protrusions seal the cavity. Encapsulation of the substrate is not accompanied by its release into the cavity; rather, it remains bound to the chaperonin (state *b*). The energy of ATP binding (and possibly hydrolysis) is further used to force the folding of substrates, whereby the open conformations of actin and tubulin change into compact, quasi-native (or native) structures that are not liberated into the CCT cavity but remain bound to the chaperonin (state *c*). Transition into state *d* is thought to depend on the hydrolysis of ATP; the mechanism of the putative allosteric signal, which would trigger the opening of the cavity and release of the folded substrate (N), remains unknown. The figure only shows the functioning of a single CCT ring; interactions between rings are not considered. A, apical domain; I, intermediate domain; E, equatorial domain. Taken from [75] and [86] with alterations. See text for details.

liberated into the CCT cavity. Therefore, a fundamental difference exists between the mechanisms employed by GroEL and CCT at the stage following the closure of the cavity.

As distinct from GroEL, the eucaryotic chaperonin does not sever its connection with the substrate, keeping it as part of a complex, which alters its structure in the course of folding to the native conformation. According to the concept developed by the Madrid group [84], CCT employs an active, physical mechanism whereby the movements of apical domains upon ATP binding are coupled to the folding movements of actin and tubulin. It is proposed that the binding of ATP to different subunits of the CCT ring, which is positively cooperative, occurs sequentially in the order CCTα → CCTη → CCTδ → CCTθ → CCTγ → CCTβ → CCTζ → CCTε, i.e., anti-clockwise, as shown in Fig. 5 (*c*, the outer ring).

The subunits that are the first to bind ATP are those involved in the binding of the N-terminal domains of the substrate proteins (the δ subunit in the case of actin, and the α and η subunits in the case of tubulin, see Fig. 5). It is hypothesized that the sequential anti-clockwise conformational changes undergone by the apical domains upon ATP binding (and possibly its hydrolysis), first reach the N-terminal domains of actin and tubulin, causing their liberation from the chaperonin and thus freeing them to move towards the C-terminal domains. In so doing, they shift clockwise relative to the corresponding C-terminal domains that remain firmly bound to the chaperonin (Fig. 5*c*, the inner ring).

The substrate protein acquires a native-like conformation, characterized by the existence of correct interdomain interactions. This is shown schematically in Fig. 5 (state *d*). In this manner, the circular structure of the CCT ring, with eight different subunits arranged in a precise order, gives rise to a vectorial mechanism that couples the conformational changes in the apical domains of the chaperonin subunits to concerted movements in the substrate molecules that lead to their successful folding.

Turning back to Fig. 4, it should be noted that the scheme of CCT functioning is still largely hypothetical. The following important points are not sufficiently clear and are open to discussion. Is the closing of the CCT cavity achieved using ATP binding energy, as in the case of GroEL, or does it take place only as a result of ATP hydrolysis [85]? Is the folding of the substrate protein accomplished before its release inside the cavity (as shown in Fig. 4)? It also remains to be elucidated exactly what triggers the release of the substrate into the cavity (although most authors share the view that the energy of ATP hydrolysis should be used for that purpose). It is also suggested that the opening of the cavity (state *e*) should occur in response to an allosteric signal from the neighboring ring, as is the case in GroEL. Further studies are needed to clarify these points.

The question of whether a single cycle of CCT functioning is sufficient for complete substrate folding has been the subject of considerable attention in recent years. In a number of studies by Hartl's laboratory results were obtained in support of the notion that CCT captures actin

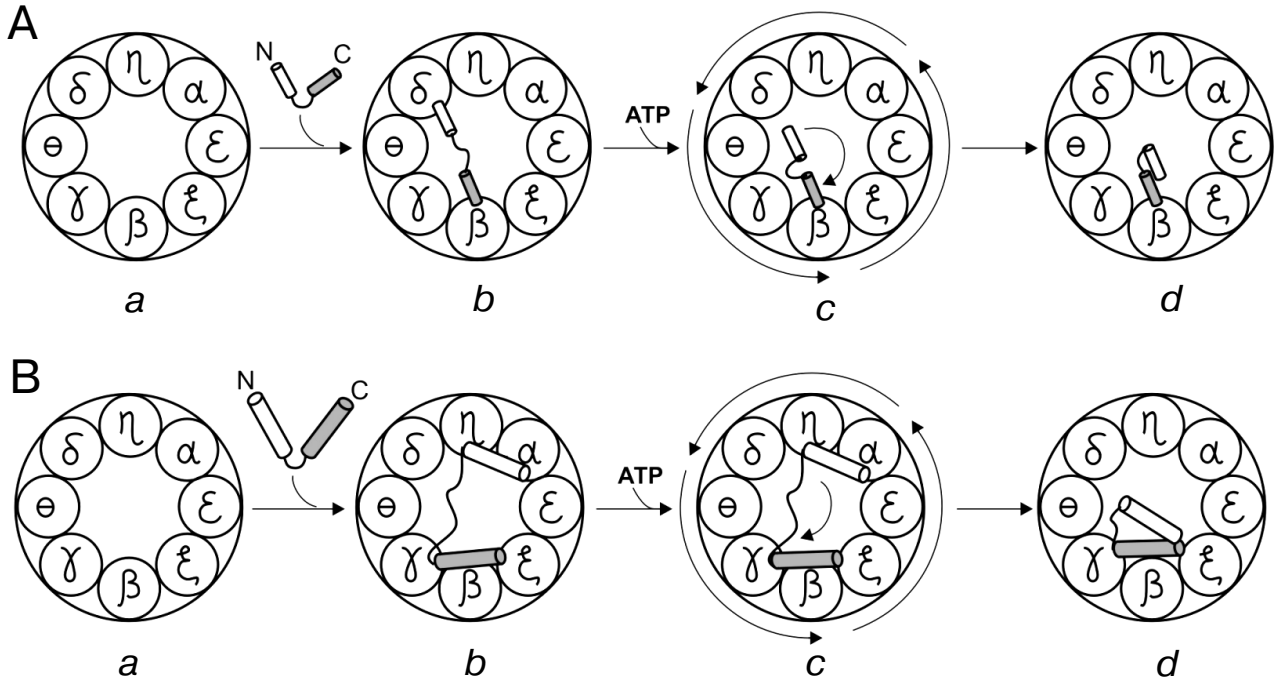

Fig. 5. Suggested mechanism of CCT participation in the folding of actin (A) and tubulin (B). *a*) Schematic representation of the CCT ring, comprising eight different subunits (top view); *b*) binding of a substrate protein. The binding of actin, whose N- and C-terminal domains are represented by white and gray cylinders, respectively, involves the participation of two subunits: either δ and ε or δ and β; the second case is shown. The N-terminal domain of tubulin can be bound either by the η and α subunits (as shown in the figure), or by the θ and β subunits, whereas the C-terminal domain can be bound by the γ, β, and ζ or by the ε, ζ, and β subunits; the figure shows the first of these two possibilities. The binding of ATP to different CCT subunits is positively cooperative and occurs sequentially in the order CCTα → CCTη → CCTδ → CCTθ → CCTγ → CCTβ → CCTζ, etc., i.e., anti-clockwise, as shown in the figure (*c*, the outer ring). For this reason, conformational changes induced by the binding of ATP and by its hydrolysis primarily affect the apical domains of subunits that bind the N-terminal domains of the substrate protein. Upon release from the chaperonin, the N-terminal domains are able to change their position by moving clockwise, as shown in the figure (*c*, the inner ring), in relation to the corresponding C-terminal domains that remain firmly bound to the chaperonin. The substrate protein acquires a native-like conformation (state *d*). Further details are provided in the text. Taken from [84] with alterations.

directly from the translating ribosomes and facilitates its folding during a single functional cycle [93-98]. This becomes possible due to a specific set of protein–protein interactions between separate components of the "folding machine" in the cell.

Thus, newly synthesized N-terminal domain of actin interacts first with a co-chaperonin, prefoldin (in mammalian cells) or GimC (in yeasts), and is then passed on to the chaperonin CCT [99, 100]. In experiments carried out with yeasts, it has been established that the presence of GimC accelerates the folding of actin at least by a factor of 5 while preventing premature release of misfolded forms from their complex with CCT, which considerably increases the yield of folded protein [98]. Both these effects appeared to arise from a direct influence of the co-chaperonin on the properties of the binary CCT–actin complex.

These results were interpreted as evidence for the existence of an isolated compartment wherein CCT and GimC perform their coordinated role in actin folding. The ability of GimC to simultaneously bind both CCT and non-native forms of actin provides means to confine these forms to the isolated compartment, preventing their egress outside. A more intriguing effect discovered in this study was the acceleration of protein folding induced by the binding of GimC. The mechanism causing this effect, which may involve a change in CCT conformation, is still unknown. Future studies on this subject may provide useful insights into the mechanisms that regulate the folding process in the cell.

STERIC CHAPERONES. ACCELERATING THE FINAL STEPS OF PROTEIN FOLDING

The steric or intramolecular chaperones constitute a special group playing a key role in the folding of a large number of proteins The name of these chaperones reflects their distinctive features, which can be summarized as follows. Steric chaperones can accelerate the folding of other proteins by interacting with their folding intermediates and thereby conveying to them steric information

essential for folding. In this manner, they directly catalyze the folding process. In the case of bacterial extracellular proteases, the information is inherent in the amino acid sequence of a pro-domain that is autocatalytically cleaved off the mature protein by limited proteolysis. In the case of lipases of Gram-negative bacteria, an individual protein fulfils the role of a steric chaperone, functioning in a way similar to the pro-domains of bacterial proteases.

The most detailed studies on the properties of steric chaperones and their mechanism of action have been performed with bacterial serine proteinases, particularly subtilisin. This protein, produced as a precursor, pre-pro-subtilisin [101], is secreted as pro-subtilisin (352 amino acid residues), containing a pro-domain (77 residues). The maturation of this enzyme involves several steps: folding of pro-subtilisin, autoprocessing of pro-subtilisin from the N-terminus accompanied by the formation of a binary {inactive subtilisin·pro-domain} complex, formation of active subtilisin, and degradation of the cleaved pro-domain [102, 103].

The following experiment has been used to demonstrate that the pro-domain promotes the refolding of the mature subtilisin sequence in an intermolecular process. The 275-amino acid mature sequence was produced in *E. coli* in an inactive form and then dialyzed against 6 M guanidine hydrochloride to ensure complete unfolding. After the denaturant was removed by dialysis, no activity was restored and the protein remained stabilized in a compact, molten globule-like structure (its further folding being an extremely slow process [104]). However, when the pro-domain sequence was added to the protein solution, subtilisin activity was recovered in proportion to the concentration of added pro-domain [105-109]; in this process, the folding rate was increased by several orders of magnitude [110].

An explanation of the mechanism used by the pro-domain to dramatically accelerate subtilisin folding was offered based on the following experimental evidence. First of all, it was established that the noncovalent complex formed following the rupture of the bond between the pro-domain and subtilisin is extremely tight, with a K_a of $2 \cdot 10^8$ M^{-1} at 25°C [111]. Given that the pro-domain, which is a potent competitive inhibitor of the active subtilisin ($K_i = 5.4 \cdot 10^{-7}$ M), should possess a high affinity to the native enzyme conformation [112], it was suggested that its binding to subtilisin that has been "frozen" in one of the final stages of folding, would favor the formation of a native-like structure.

The second step was to determine the three-dimensional structure of the binary complex between the pro-domain and subtilisin [110], in order to obtain information on the structural determinants on the subtilisin surface that are involved in interaction with the pro-domain. It was found that the pro-domain, largely unstructured by itself, folds into a compact structure with a four-stranded antiparallel β-sheet and two three-turn α-helices when complexed with subtilisin (see Fig. 6a).

Figure 6b shows that the β-sheet of the pro-domain packs tightly against the two parallel surface α-helices of subtilisin. Two proline residues located in the C-terminal end of the pro-domain form "caps" for the N-termini of the two subtilisin helices, while the other C-terminal residues extend outward from the central part of the pro-domain and bind in a substrate-like manner along the active site cleft of subtilisin. Almost all of the contacts of the pro-domain with subtilisin are made by residues 100-144 [110].

These observations led the authors to conclude that the simplest model of catalyzed folding is one in which the binding interaction observed in the complex acceler-

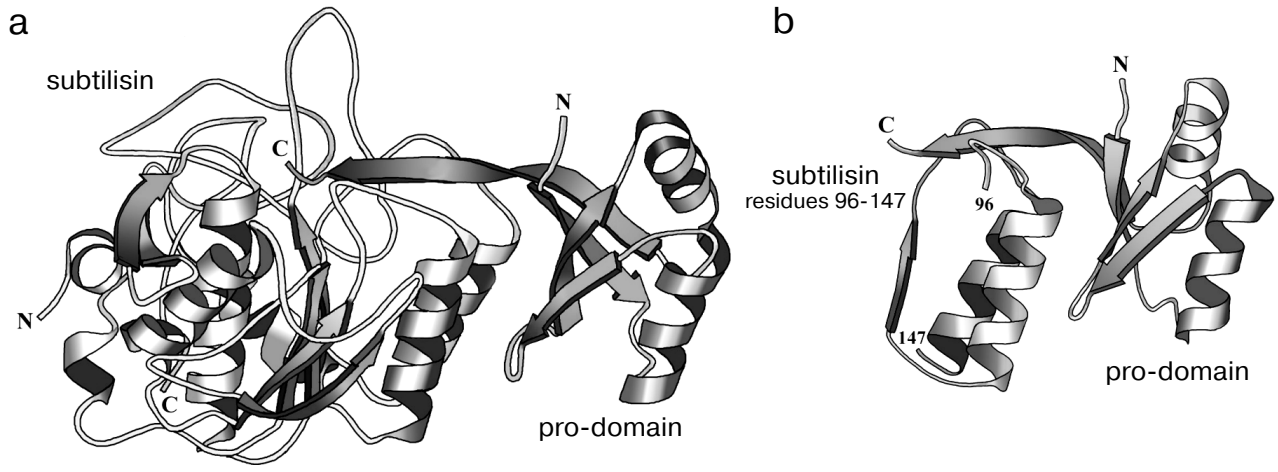

Fig. 6. a) Three-dimensional structure of the {pro-domain·subtilisin} complex. The C-terminus of the pro-domain is in the subtilisin active site. b) Pro-domain interactions with the αβα substructure in subtilisin. See text for details. Taken from [110] with alterations.

ates the folding by stabilizing the 45-amino acid αβα motif formed by residues 96-147 of subtilisin (Fig. 6b). They hypothesized that the αβα substructure, whose formation is a rate limiting step in the folding process, may act as a nucleus, with subsequent folding propagating into the N- and C-terminal regions of subtilisin.

What would then be the specific features of the folding pathway of subtilisin (and also of a number of other proteins with similar behavior [113]) that make the presence of steric chaperones an absolute requirement for successful folding? The answer to this question probably lies in the very low stability of the folding intermediates, which was found to be close to that of unfolded or misfolded states [110, 114-116]. Meanwhile, according to present views, an effective folding pathway implies that productive intermediates must be significantly more stable than the surrounding landscape of unfolded and misfolded conformations. The absence of such intermediates would create a high entropic barrier to folding, which has to be overcome [117]. Coming back to the structure of subtilisin (Fig. 6), one could suggest that during the folding of this protein in the absence of the pro-domain the αβα motif is not stable enough to serve as a nucleus for successful folding of the molecule into its native conformation. By stabilizing this substructure, the pro-domain ensures the formation of a stable intermediate, thereby accelerating the folding process.

The accelerating effect of steric chaperones may be important for the folding of other types of proteins that are needed, for one reason or another, to stabilize particular conformational states in the folding path. Thus, as early as in 1993, it was discovered that the extracellular lipase of *Pseudomonas cepacida* possesses a steric chaperone of its own, termed Lim, which is encoded by a gene located in the same operon as the lipase structural gene. Refolding of denatured lipase into its active conformation depends on the presence of Lim [118]. At present, many Gram-negative bacteria are known to require steric chaperones named "lipase-specific foldases" (Lif) to fold into native conformations. As in the case of the subtilisin pro-domain, the presence of Lif helps lipase to overcome an energetic barrier in the course of folding [119].

CONCLUDING REMARKS

The majority of investigations considered above were performed in the last 10 years; more than one third of them are indeed very recent. This indicates that the aspect of protein folding discussed in this review can be considered as one of the "hottest" areas in current research. There is little doubt that within the next few years significant amounts of new information will be obtained that will not only enlarge existing knowledge but possibly give rise to new concepts. First and foremost, this applies to molecular mechanisms responsible for the participation of eucaryotic chaperonin CCT in the folding of proteins with complicated three-dimensional structures. This is presently the focus of attention of several laboratories [84, 85].

It can be anticipated that similar problems will become the subject of studies into the action mechanism of chaperone Hsp90, in which the most important stage appears to be a conformational rearrangement of the substrate protein confined in a closed hollow formed by this chaperone with the participation of Hsp70 and a number of partner proteins [120]. Like the eucaryotic chaperonin, Hsp90 does not take part in nascent chain folding. The native targets of Hsp90 are signaling proteins in their incompletely folded or misfolded conformations; Hsp90 participates in the final stages of their folding. The transition from an incompletely folded state to a functionally competent conformation requires the energy of ATP hydrolysis [121, 122], suggesting an interrelation between the functioning of chaperone Hsp90 and the efficiency of the signal transduction processes [123, 124].

In conclusion, it is worth mentioning that detailed studies devoted to steric chaperones have contributed significantly to our present knowledge of protein folding. First of all, these studies have demonstrated that the information encoded in the amino acid sequence of a particular protein may not be sufficient to ensure its folding into the native conformation. This discovery, documented by experimental evidence that the presence of an external factor is necessary for the folding of some proteins (e.g., subtilisin and bacterial lipases), opened up a new chapter in the study of catalyzed protein folding and its regulation in the cell.

Second, as has been demonstrated in the elegant studies of Shinde and coauthors, introducing point mutations in the pro-domain leads to the folding of subtilisin into several enzymatically active conformations, which differ in their physicochemical properties [125, 126]. This suggests an interesting possibility to influence the final stages of folding by altering the structure of a steric chaperone, and offers fresh opportunities for future studies.

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REFERENCES

1. Anfinsen, C. B. (1973) *Science*, **181**, 223-230.
2. Dobson, C., and Karplus, M. (1999) *Curr. Opin. Struct. Biol.*, **9**, 92-101.
3. Coyle, J. E., Jaeger, J., Gross, M., Robinson, C. V., and Radford, S. E. (1997) *Struct. Fold. Des.*, **2**, R93-R104.
4. Bukau, B., and Horwich, A. L. (1998) *Cell*, **92**, 351-366.
5. Gutsche, L., Erssen, L. O., and Baumeister, W. (1999) *J. Mol. Biol.*, **293**, 295-312.
6. Fewell, S. W., Travers, K. J., Weissman, J. C., and Brodsky, J. L. (2001) *Annu. Rev. Genet.*, **35**, 149-191.

7. Hartl, F. U., and Hayer-Hartl, M. (2002) *Science*, **295**, 1852-1858.
8. Balbach, J., and Schmid, F. X. (2000) in *Mechanisms of Protein Folding* (Pain, R. H., ed.) IRL Press, Oxford, pp. 212-249.
9. Wu, Y., and Matthews, C. R. (2002) *J. Mol. Biol.*, **322**, 1-13.
10. Creighton, T. E. (2000) in *Mechanisms of Protein Folding* (Pain, R. H., ed.) IRL Press, Oxford, pp. 250-278.
11. Frand, A. R., Cuozzo, J. W., and Kaiser, C. A. (2000) *Trends Cell Biol.*, **10**, 203-210.
12. Ellis, R. J., and Hartl, F. U. (1996) *FASEB J.*, **10**, 20-26.
13. Todd, M. J., Lorimer, G. H., and Thirumalai, D. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 4030-4035.
14. Corrales, F. J., and Fersht, A. R. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 4509-4512.
15. Sosnick, T. R., Mayne, L., Hiller, R., and Englander, S. W. (1994) *Nature Struct. Biol.*, **1**, 149-156.
16. Shtilerman, M., Lorimer, G., and Englander, S. W. (1999) *Science*, **284**, 822-825.
17. Fayet, O., Ziegelhoffer, T., and Georgopoulos, C. (1989) *J. Bacteriol.*, **171**, 1379-1385.
18. Horwich, A. L., Low, K. B., Fenton, W. A., Hirshfield, N. L., and Furtak, K. (1993) *Cell*, **74**, 909-917.
19. Houry, W. A., Frishman, D., Eckerskom, C., Lottspeich, F., and Hartl, F. U. (1999) *Nature*, **402**, 147-154.
20. Braig, K., Otwinowcki, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L., and Sigler, P. B. (1994) *Nature*, **371**, 578-586.
21. Xu, Z., Horwich, A. L., and Sigler, P. B. (1997) *Nature*, **388**, 741-750.
22. Roseman, A. M., Chen, S. X., White, H., Braig, K., and Sabil, H. R. (1996) *Cell*, **87**, 241-251.
23. Fenton, W. A., Kashi, Y., Furtak, K., and Horwich, A. L. (1994) *Nature*, **371**, 614-619.
24. Farr, G. W., Furtak, K., Rowland, M. B., Ranson, N. A., Sabil, H. R., Kirchhausen, T., and Horwich, A. L. (2000) *Cell*, **100**, 561-573.
25. Hunt, J. F., Weaver, A. J., Landry, S. L., Gierasch, L., and Deisenhofer, J. (1996) *Nature*, **379**, 37-45.
26. Saibil, H. R., Kirchhausen, T., and Horwich, A. L. (2000) *Cell*, **100**, 561-573.
27. Yifrach, O., and Horovitz, A. (1994) *J. Mol. Biol.*, **243**, 397-401.
28. Yifrach, O., and Horovitz, A. (1994) *Biochemistry*, **34**, 5303-5308.
29. Hayer-Hartl, M. K., Martin, J., and Hartl, F. U. (1995) *Science*, **269**, 836-841.
30. Weissman, J. S., Hohl, C. M., Kovalenko, O., Kashi, Y., Chen, S. X., Braig, K., Saibil, H. R., Fenton, W. A., and Horwich, A. L. (1995) *Cell*, **83**, 577-587.
31. Mayhew, M., Da Silva, A. C. R., Martin, J., Erdjument-bromage, H., Tempst, P., and Hartl, F. U. (1996) *Nature*, **379**, 420-426.
32. Weissman, J. S., Rye, H. S., Fenton, W. A., Beechem, J. M., and Horwich, A. L. (1996) *Cell*, **84**, 481-490.
33. Wang, J., and Boisvert, D. C. (2003) *J. Mol. Biol.*, **327**, 843-855.
34. Sakikawa, G., Tagushi, H., Makino, Y., and Yoshida, M. (1999) *J. Biol. Chem.*, **274**, 21251-21256.
35. Ranson, N. A., Burston, S. G., and Clarke, A. R. (1997) *J. Mol. Biol.*, **266**, 656-664.
36. Yifrach, O., and Horovitz, A. (1995) *Biochemistry*, **34**, 5303-5308.
37. Yifrach, O., and Horovitz, A. (1996) *J. Mol. Biol.*, **255**, 356-361.
38. Ellis, R. J., and Hartl, F. U. (1999) *Curr. Opin. Struct. Biol.*, **9**, 102-110.
39. Saibil, H. R., and Ranson, N. A. (2002) *Trends Biochem. Sci.*, **27**, 627-632.
40. Ranson, N. A., Dunster, N. J., Burston, S. G., and Clarke, A. R. (1995) *J. Mol. Biol.*, **250**, 581-586.
41. Smith, K. E., and Fisher, M. T. (1995) *J. Biol. Chem.*, **270**, 21517-21523.
42. Taguchi, H., and Yoshida, M. (1995) *FEBS Lett.*, **359**, 195-198.
43. Peralta, D., Hartman, D. J., Hoogenraad, N. J., and Hoj, P. B. (1994) *FEBS Lett.*, **339**, 45-49.
44. Sparrer, H., Rutkat, K., and Buchner, J. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 1096-1000.
45. Todd, M. J., Vitanen, P. V., and Lorimer, G. H. (1994) *Science*, **265**, 659-666.
46. Dill, K. A., and Chan, H. S. (1997) *Nature Struct. Biol.*, **4**, 10-19.
47. Weissman, J. S., and Kim, P. S. (1991) *Science*, **253**, 1386-1393.
48. Todd, M. J., Lorimer, G. H., and Thirumalai, D. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 4030-4035.
49. Corrales, F. J., and Fersht, A. R. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 4509-45112.
50. Goluoubinoff, P., Gatenby, A. A., and Lorimer, G. H. (1989) *Nature*, **337**, 44-47.
51. Goluoubinoff, P., Cristeller, J. T., Gatenby, A. A., and Lorimer, G. H. (1989) *Nature*, **342**, 884-889.
52. Walter, S., Lorimer, F. X., and Schmid, F. X. (1996) *Proc. Natl. Acad. USA*, **93**, 9425-9430.
53. Wang, J. D., and Weissman, J. S. (1999) *Nature Struct. Biol.*, **6**, 597-600.
54. Brinker, A., Pfeifer, G., Kerner, M. J., Naylor, D. J., Hartl, F. U., and Hayer-Hartl, M. (2001) *Cell*, **19**, 223-233.
55. Rye, H. S., Burston, S. G., Fenton, W. F., Beechem, J. M., Xu, Z., Sigler, P. B., and Horwich, A. L. (1997) *Nature*, **388**, 792-798.
56. Hayer-Hartl, M. K., Weber, F., and Hartl, F. U. (1996) *EMBO J.*, **15**, 6111-6121.
57. Wolynes, P. G., Onuchik, J. N., and Thirumalai, D. (1995) *Science*, **267**, 1619-1620.
58. Persson, M., Carlsson, U., and Bergenhem, N. (1997) *FEBS Lett.*, **411**, 43-47.
59. Coyle, J. E., Texter, F. L., Ashcroft, A. E., Masselos, D., Robinson, C. V., and Radford, S. E. (1999) *Nature Struct. Biol.*, **6**, 683-690.
60. Radford, S. E., and Dobson, C. M. (1995) *Phil. Trans. R. Soc. Lond.* B, **348**, 17-25.
61. Miranker, Radford, S. E., Karplus, M., and Dobson, C. M. (1991) *Nature*, **349**, 633-636.
62. Radford, S. E., Dobson, C. M., and Evans, P. A. (1992) *Nature*, **358**, 302-307.
63. Matagne, A., Radford, S. E., and Dobson, C. M. (1997) *J. Mol. Biol.*, **267**, 1068-1074.
64. Ewalt, K. L., Hendrick, J. P., Houry, W. A., and Hartl, F. U. (1997) *Cell*, **90**, 491-500.
65. Rothwarf, D. M., and Scheraga, H. A. (1996) *Biochemistry*, **35**, 13797-13807.
66. Wildegger, G., and Kiefhaber, T. (1997) *J. Mol. Biol.*, **270**, 294-304.
67. Chan, H. S., and Dill, K. A. (1996) *Proteins: Struct. Func. Genet.*, **24**, 345-351.
68. Lorimer, G. H. (1996) *FASEB J.*, 1**0**, 5-9.
69. Rye, H. S., Roseman, A. M., Chen, S., Furtak, K., Fenton, W. A., Saibil, H. R., and Horwich, A. L. (1999) *Cell*, **97**, 325-338.

70. Wang, J. D., Herman, C., Tipton, K. A., Gross, C. A., and Weissman, J. S. (2002) *Cell*, **111**, 1027-1039.
71. Erbse, A. F., Dougan, D. A., and Bukau, B. (2003) *Nature Struct. Biol.*, **10**, 84-86.
72. Sternlicht, H., Farr, G. W., Sternlicht, M. L., Driscoll, J. K., Willison, K. R., and Yaffe, M. B. (1993) *Proc. Natl. Acad. Sci. USA*, **94**, 9422-9426.
73. Willison, K. R., and Grantham, J. (2001) in *Molecular Chaperones: Frontiers in Molecular Biology* (Lund, P., ed.) Oxford University Press, Oxford, pp. 90-118.
74. Tian, G., Vainberg, I. E., Tap, W. D., Lewis, S. A., and Cowan, N. J. (1995) *Nature*, **375**, 250-253.
75. Valpuesta, J. M., Martin-Benito, J., Gomez-Puertas, P., Carrascosa, J. L., and Willison, K. R. (2002) *FEBS Lett.*, **529**, 11-16.
76. Klumpp, M., Baumeister, W., and Essen, L.-O. (1997) *Cell*, **91**, 263-270.
77. Ditzel, L., Lowe, J., Stock, D., Stetter, K.-O., Huber, H., Huber, R., and Steinbacher, S. (1998) *Cell*, **93**, 125-138.
78. Horwich, A. L., and Saibil, H. R. (1998) *Nature Struct. Biol.*, **5**, 333-336.
79. Pappenberger, G., Wilsher, J. A., Roe, S. M., Counsell, D. J., Willison, K. R., and Pearl, L. H. (2002) *J. Mol. Biol.*, **318**, 1367-1379.
80. Nitsch, M., Walz, J., Typke, D., Klumpp, M., Essen, L.-O., and Baumeister, W. (1998) *Nat. Struct. Biol.*, **5**, 855-857.
81. Llorka, O., McCormack, E., Hynes, G. M., Grantham, J., Cordell, J., Carrascosa, J. L., Willison, K. R., Fernandez, J. J., and Valpuesta, J. M. (1999) *Nature*, **402**, 693-696.
82. Llorka, O., Smyth, M. G., Carrascosa, J. L., Willison, K. R., Radermacher, M., Steinbacher, S., and Valpuesta, J. M. (1999) *Nat. Struct. Biol.*, **6**, 639-642.
83. Llorka, O., Benito-Martin, J., Ritko-Vonsovici, M., Grantham, J., Hynes, G. M., Willison, K. R., Carrascosa, J. L., and Valpuesta, J. M. (2000) *EMBO J.*, **19**, 5971-5979.
84. Llorca, O., Martin-Benito, J., Grantham, J., Ritco-Vonsovici, M., Willison, K. R., Carrascosa, J. L., and Valpuesta, J. M. (2001) *EMBO J.*, **20**, 4065-4075.
85. Meyer, A. S., Gillespie, J. R., Walther, D., Millet, J. S., Doniach, S., and Frydman, J. (2003) *Cell*, **113**, 369-381.
86. Kusmierczyk, A. R., and Martin, J. (2001) *FEBS Lett.*, **505**, 343-347.
87. Willison, K. R. (1999) in *Molecular Chaperones and Folding Catalysis* (Bukau, B., ed.) Harwood Academic Publishers, pp. 555-571.
88. Rommelaere, H., de Neve, M., Melki, R., Vandekerckhove, J., and Ampe, C. (1999) *J. Biol. Chem.*, **38**, 3246-3257.
89. Hunes, G. M., and Willison, K. R. (2000) *J. Biol. Chem.*, **276**, 18985-18994.
90. Ritko-Vonsovici, M., and Willison, K. R. (2000) *J. Mol. Biol.*, **304**, 81-98.
91. Dobrzynski, J. K., Sternlicht, M. L., Peng, I., Farr, G. W., and Sternlicht, H. (2002) *Biochemistry*, **39**, 3988-4003.
92. Horovitz, A., Fridmann, Y., Kafri, G., and Yifrach, O. (2001) *J. Struct. Biol.*, **135**, 104-114.
93. Thulasiraman, V., Yang, C. F., and Frydman, J. (1999) *EMBO J.*, **18**, 85-95.
94. Frydman, J., Nimmesgern, E., Ohtsuka, K., and Hartl, F. U. (1994) *Nature*, **370**, 111-117.
95. Frydman, J., and Hartl, F. U. (1996) *Science*, **272**, 1497-1502.
96. Netzer, W. J., and Hartl, F. U. (1997) *Nature*, **388**, 343-349.
97. McCallum, C. D., Do, H., Johnson, A. E., and Frydman, J. (2000) *J. Cell. Biol.*, **149**, 591-602.
98. Siegers, K., Waldmann, T., Leroux, M. R., Grein, K., Shevchenko, A., Schiebel, E., and Harll, F. U. (1999) *EMBO J.*, **18**, 75-84.
99. Geissler, S., Siegers, K., and Schiebel, E. (1998) *EMBO J.*, **17**, 952-966.
100. Vainberg, I. E., Lewis, S. A., Rommerlaere, H., Ampe, C., Vandekerckhove, J., Klein, H. L., and Cowan, N. J. (1998) *Cell*, **93**, 863-873.
101. Wells, J. A., Ferrari, E., Henner, D. J., Estell, D. A., and Chen, E. Y. (1983) *Nucleic Acids Res.*, **11**, 7911-7925.
102. Power, S. D., Adams, R. M., and Wells, J. A. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 3096-3100.
103. Ikemura, H., Takagi, H., and Inouye, M. (1987) *J. Biol. Chem.*, **262**, 7859-7864.
104. Bryan, P., Alexander, P., Strausberg, S., Schwarz, F., Wang, L., Gilliland, G., and Gallagher, D. T. (1992) *Biochemistry*, **31**, 4937-4945.
105. Zhu, X., Ohta, Y., Jordan, F., and Inouye, M. (1989) *Nature*, **339**, 483-484.
106. Silen, J. L., and Agard, D. A. (1989) *Nature*, **341**, 462-464.
107. Shinde, U., Li, Y., Chatterjee, S., and Inouye, M. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 6924-6928.
108. Shinde, U., and Inouye, M. (1993) *Trends Biochem. Sci.*, **18**, 442-446.
109. Ellis, J. R. (1998) *Trends Biochem. Sci.*, **23**, 43-45.
110. Bryan, P., Wang, L., Hoskins, J., Ruvinov, S., Strausberg, S., Alexander, P., Almog, O., Gilliland, G., and Gallagher, D. T. (1995) *Biochemistry*, **34**, 10310-10318.
111. Strausberg, S., Alexander, P., Wang, L., Schwarz, F., and Bryan, P. (1993) *Biochemistry*, **32**, 8112-8119.
112. Bryan, P. N. (2002) *Chem. Rev.*, **102**, 4805-4815.
113. Baker, D., and Agard, D. (1994) *Biochemistry*, **33**, 7505-7509.
114. Wang, L., Ruvinov, S., Strausberg, S., Gallagher, D. T., Gilliland, G., and Bryan, P. (1995) *Biochemistry*, **34**, 15415-15420.
115. Wang, L., Ruan, B., Ruvinov, S., and Bryan, P. N. (1998) *Biochemistry*, **37**, 3165-3171.
116. Ruan, B., Hoskins, J., and Bryan, P. (1999) *Biochemistry*, **38**, 8562-8571.
117. Levinthal, C. (1968) *J. Chim. Phys.*, **65**, 44-45.
118. Hobson, A. H., Buckley, C. M., Aamand, J. L., Jorgensen, S. T., Diderichsen, B., and McConnell, D. J. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 5682-5686.
119. El Khattabi, M., van Gelder, P., Bitter, W., and Tommassen, J. (2000) *J. Biol. Chem.*, **275**, 26885-26891.
120. Young, J. C., Moarefi, I., and Hartl, F. U. (2001) *J. Cell. Biol.*, **154**, 267-273.
121. Obermann, W. M. J., Sondermann, H., Russo, A. A., Pavletich, N. P., and Hartl, F. U. (1998) *J. Cell. Biol.*, **143**, 901-910.
122. Panaretou, B. C., Prodromou, S. M., Roe, R., O'Brien, J. E., Ladbury, P. W., Piper, P. W., and Pearl, L. H. (1998) *EMBO J.*, **17**, 4829-4836.
123. Mayer, M. P., Nicilay, R., and Bukau, B. (2002) *Mol. Cell*, **10**, 1255-1268.
124. Pearl, L. H., and Prodromou, S. (2001) *Adv. Prot. Chem.*, **59**, 157-186.
125. Shinde, U. P., Liu, J. J., and Inouye, M. (1997) *Nature*, **389**, 520-522.
126. Shinde, U. P., and Inouye, M. (1999) *J. Biol. Chem.*, **274**, 15615-15621.