Requirement for GroEL/GroES-Dependent Protein Folding under Nonpermissive Conditions of Macromolecular Crowding†

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ABSTRACT: Macromolecular crowding is a critical parameter affecting the efficiency of cellular protein folding. Here we show that the proteins dihydrofolate reductase, enolase, and green fluorescent protein, which can fold spontaneously in diluted buffer, lose this ability in a crowded environment. Instead, they accumulate as soluble, protease-sensitive non-native species. Their folding becomes dependent on the complete GroEL/GroES chaperonin system and is not affected by trap-GroEL, indicating that folding has to occur in the chaperonin cavity with release of nativelike proteins into the bulk solution. In addition, we demonstrate that efficient folding in the chaperonin cavity requires ATP hydrolysis, as formation of ternary GroEL/GroES complexes with substrate proteins in the presence of ADP results only in very inefficient reactivation. However, protein refolding reactions using ADP−fluoroaluminate complexes, or single-ring GroEL and GroES under conditions where only a single round of ATP hydrolysis occurs, yield large amounts of refolded enzymes. Thus, the mode of initial ternary complex formation appears to be critical for subsequent productive release of substrate into the cavity under certain crowding conditions, and is only efficient when triggered by ATP hydrolysis. Our data indicate that stringent conditions of crowding can impart a stronger dependence of folding proteins on the assistance by chaperonins.

Newly synthesized proteins that are folding in the cytosol face a unique physicochemical environment. The concentration of nascent ribosome-bound polypeptide chains in the cytosol of *Escherichia coli* is as high as ∼35 μM, assuming a uniform distribution (*1*). However, their effective concentration will be significantly higher because of macromolecular crowding. This term refers to the fact that a considerable fraction of the cellular volume is occupied by protein and other macromolecules at a total concentration of more than 300 g/L, and is therefore not available to other macromolecules (*2*, *3*). The significance of macromolecular crowding for protein folding has been only recently addressed. For example, for reduced lysozyme, it was observed that crowding changed the rates but not substantially the energetics of folding and that protein disulfide isomerase was an effective catalyst of its folding in crowding agents (*4*). Earlier studies had already shown that the folding helpers GroEL and GroES are active under these conditions, too (*5*). The GroEL/GroES chaperonin system prevents the aggregation of newly synthesized polypeptides and promotes their efficient folding in the bacterial cytosol (*6*−*8*). GroEL is composed of two heptameric rings of ∼57 kDa subunits stacked back to back, each ring containing hydrophobic binding regions for unfolded polypeptides that face the central cavity. GroES is a single ring of seven ∼10 kDa subunits. The mechanistic principles of chaperonin action have been established (*6*, *9*, *10*). Unfolded polypeptide binds to an asymmetrical GroEL/GroES complex within the cavity of the GroEL ring (cis) that is not occupied by GroES, followed by the dissociation of GroES. Rebinding of GroES to the cis ring of GroEL together with ATP results in the encapsulation of bound polypeptide and the burial of the hydrophobic regions of the inner surface of GroEL. Bound polypeptide is released into an enlarged GroEL cavity for folding, and the bound ATP is hydrolyzed. A subsequent round of ATP binding and hydrolysis in the trans GroEL ring triggers the dissociation of GroES from GroEL, thus opening the folding cage. While there is strong evidence that folding occurs in the chaperonin cavity, a fraction of bound polypeptide can be released from GroEL into solution during each reaction cycle (*5*, *11*). However, this release of non-native polypeptide from GroEL is typically nonproductive, and correct folding requires rebinding to the same or another chaperonin molecule (*12*). In contrast to these in vitro conditions, the level of apparent leakage of the chaperonin system was found to be strongly reduced when folding of the stringent substrates rhodanese and malate dehydrogenase (MDH)[1] was carried out in the presence of crowding agents (*5*). Neither kinetics nor yields of folding were negatively affected by these conditions. These results led to the conclusion that intermittent release of unfolded polypeptide into the bulk solution is not required

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[1] Abbreviations: MDH, malate dehydrogenase; GFP, green fluorescent protein; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DHFR, dihydrofolate reductase; GdmCl, guanidinium chloride; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate; ADP−AlFn, ADP−aluminum-fluoride complex; PDI, protein disulfide isomerase.

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for GroEL/GroES-mediated folding. An ensuing question is whether this tighter coupling of folding and release under conditions of macromolecular crowding applies also to proteins that, unlike rhodanese and MDH, are not GroES-dependent in diluted buffer systems. To address this question, we tested chaperonin-mediated protein folding of several monomeric and oligomeric proteins in Ficoll 70, a crowding agent that is frequently used to examine excluded volume effects on protein renaturation (*13*−*15*). We present data demonstrating that under stringent conditions of crowding spontaneous folding yields decrease and that efficient chaperonin-mediated folding requires ATP hydrolysis. Concomitantly, chaperonin-mediated protein folding becomes increasingly GroES-dependent. Our data underline the importance of folding helper proteins and catalysts under circumstances that pose a challenge to protein folding.

EXPERIMENTAL PROCEDURES

Protein Expression, Purification, and Modification. GroEL, single-ring GroEL, mutant protein GroEL337/349, GroES, and recombinant GFP were purified as described previously (*5*, *16*−*18*). Trap GroEL, internally cross-linked with glutaraldehyde (GA-GroEL), was prepared according to published protocols (*16*). Mouse DHFR, yeast enolase, and bovine liver rhodanese were obtained from Sigma. Protein concentrations were determined by the Bradford assay and spectrophotometrically using the procedure outlined by Gill and von Hippel (*18*, *19*).

Protein Refolding in Aqueous Solutions and in the Presence of Crowding Agents. Substrate proteins (5.4 μM DHFR, 80 μM rhodanese, 20 μM GFP, and 25 μM enolase) unfolded in 5.3 M GdmCl and 1 mM DTT were bound to GroEL or single-ring GroEL by 150-fold dilution either into buffer A (25 mM MOPS-NaOH, 50 mM KCl, 5 mM $MgCl_2$, and 3 mM DTT) or buffer B for rhodanese (buffer A with 50 mM sodium thiosulfate). Samples contained in each case a 3-fold molar excess of GroEL. Reactions including single-ring GroEL were also carried out under low-salt conditions (5 mM KCl) which leads to arrest of ternary complexes in the ADP state. All experiments were performed at 25 °C. Where indicated, samples received trap-GroEL (GA-GroEL or mutant GroEL337/349) at the indicated concentrations. Refolding was initiated by adding 3 mM ATP, AMP-PNP, the ADP−fluoroaluminate complex (ADP−AlFn), or ADP and GroES at a 2-fold molar excess over total GroEL. The transition state analogue ADP−AlFn was generated as previously described (*20*).

Activity and Conformation Assays. The fluorescence of native GFP was measured as described previously (*17*, *18*). Enzymatic activities of DHFR, rhodanese, and enolase were measured using published procedures (*21*, *22*). The final concentration of Ficoll 70 in the enzyme assays (8%) was without effect. To assess the conformation of spontaneously refolding protein species in buffer and Ficoll 70, GdmCl-denatured proteins were diluted 150-fold into buffer A or 28% Ficoll 70 and allowed to refold for 45 min. At this time, each sample was split. One part was tested for sensitivity to proteinase K (PK) by incubating samples with 5 μg/mL PK for 10 min at 25 °C, followed by incubation with 1 mM phenylmethanesulfonyl fluoride for 5 min. Subsequently, samples were analyzed by SDS−PAGE and staining with Coomassie Blue (*21*). The other part was tested for the possible formation of high-molecular weight aggregates in the presence of crowding agents by centrifugation, as previously described (*4*). Measurements of GroEL ATPase activity were carried out according to published procedures (*21*).

RESULTS AND DISCUSSION

When GroES-dependent substrate proteins are refolded in buffer with GroEL, GroES, and ATP, in each reaction cycle a fraction of the substrate protein is displaced into the bulk solution in a non-native conformation (*5*, *11*, *23*). While these molecules may be able to rebind to GroEL for another attempt at folding in the confined chaperonin cavity, rebinding is prevented in the presence of trap-GroEL. This modified chaperonin complex is able to bind substrate proteins, but unable to release them. Consequently, in the presence of trap-GroEL refolding yields of the substrate protein decrease (*5*, *24*). In contrast, we have previously shown that trap-GroEL has no effect on refolding yields of ATP- and GroES-dependent substrates when the reaction proceeds in the crowding agent Ficoll 70 (*5*), even though trap-GroEL retains its ability to bind non-native substrate proteins. We concluded that under crowding conditions the GroEL/GroES chaperonin system is more tightly coupled with little release of non-native proteins into the bulk solution. However, with ATP alone, GroES-dependent proteins such as rhodanese and MDH can be released in Ficoll 70 from GroEL, as shown by their ensuing association with trap-GroEL. This indicated that release of substrate protein per se functions normally in the presence of the crowding agent. How then does chaperonin-mediated reactivation in Ficoll 70 proceed with substrate proteins that, in diluted buffer solutions, are not ATP/GroES-dependent? How critical is the presence of both ATP and GroES in permitting their productive protein folding in a crowded environment, and do other conditions, which in diluted buffer are known to result in productive folding, work as well?

First, we tested reactivation of GroEL-bound rhodanese after addition of GroES and ADP (Figure 1). It has previously been shown that with ADP, like with ATP, a ternary complex forms which encapsulates the substrate protein in the chaperonin cavity (*25*, *26*). Moreover, in the presence of ADP, GroES is known to cycle between the GroEL-bound and free states (*16*). As described previously (*16*, *27*), kinetics of folding with ADP were markedly slower than those with ATP. In contrast to reactions in diluted buffer (Figure 1A), the rhodanese reactivation yield with ADP was only 24% in Ficoll 70 (Figure 1B), indicating that ATP hydrolysis is beneficial for formation of a stable, folding-competent ternary complex. Refolding was almost equally efficient in the presence of two forms of trap-GroEL which are known to be functional in crowding agents, the mutant GroEL337/34, and GroEL that was chemically cross-linked with glutaraldehyde (GA-GroEL) (*5*, *16*, *24*). However, in buffer the presence of trap-GroEL led to diminished yields (Figure 1A). Intermittent dissociation of GroES between reaction cycles has been observed as well in the analysis of rhodanese folding reactions with single-ring GroEL (*16*). We find that under crowded conditions, single-ring GroEL is capable of mediating efficient refolding of rhodanese, with yields

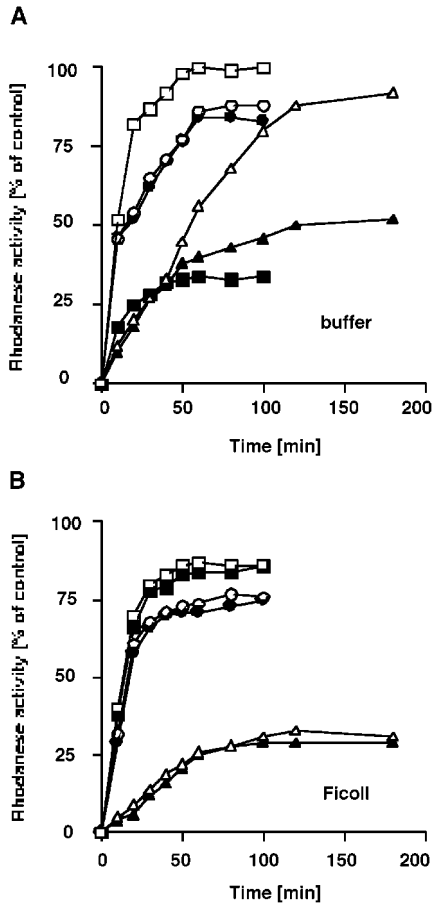

FIGURE 1: ATP hydrolysis is critical for efficient chaperonin-mediated folding of rhodanese in crowding agents. (A) Rhodanese refolding in buffer. The enzyme was refolded using the following chaperonin components in the absence (empty symbols) or presence (filled symbols) of a 5-fold molar excess of trap-GroEL: GroEL, GroES, and ATP (squares), GroEL, GroES, and ADP (triangles), and single-ring GroEL, GroES, and ATP (circles). (B) Rhodanese refolding in 28% Ficoll 70. GroEL-, GroES-, and ATP-mediated rhodanese refolding in buffer in the absence of trap-GroEL is set to 100%.

approaching those obtained with wild-type GroEL (Figure 1B). Again, trap-GroEL was without effect on this reaction, suggesting that a tighter interaction between the single-ring chaperonin and GroES took place in Ficoll 70, thus preventing escape of non-native rhodanese into solution.

Is ATP hydrolysis the key to productive folding, or is ATP binding already sufficient? We tested the nonhydrolyzable ATP analogue AMP-PNP, as well as ADP−AlFn, which mimics closely the hydrolysis transition state (*20*), on their ability to promote rhodanese refolding in buffer and Ficoll 70 together with GroEL and GroES. While both analogues were successful in aiding rhodanese folding in buffer, only the transition state analogue ADP−AlFn was an effective ligand for GroEL in Ficoll 70. Apparently, ATP binding without hydrolysis does not result in productive ternary complexes in the crowding agent or fails to induce the conformational changes in GroEL that would lead to dissociation of rhodanese from the chaperonin. The results obtained with the various nucleotides are summarized in Table 1.

The fact that GroEL in conjunction with GroES and ADP was able to mediate refolding of rhodanese at least to a certain extent without requiring ATP hydrolysis encouraged

Table 1: Final Yields of Rhodanese Reactivation in Buffer and Ficoll 70 under Various Refolding Conditions[a]

nucleotide	% refolding yield in buffer	% refolding yield in Ficoll 70
ATP	91	78
ADP	82	24
AMP-PNP	60	7
ADP−AlFn	87	77

[a] Refolding conditions were as described in Experimental Procedures. All samples contained the complete GroEL/GroES chaperonin system, in addition to the indicated nucleotide at a concentration of 1 mM. Numbers are given as the percentage of the native enzyme.

us to test nonstringent substrate proteins that can refold spontaneously in diluted buffer solutions and therefore do not depend on GroES. In buffer, in the presence of ATP alone, they are released from GroEL into the bulk solution where they can fold on their own (*17*, *21*, *22*). With GroES present, they are retained for folding in the chaperonin cavity. As described above for rhodanese, either ATP or ADP can serve as the nucleotide source in promoting productive ternary complex formation of these substrate proteins with GroEL and GroES. Several monomeric and oligomeric substrate proteins were tested for their folding characteristics in the presence of crowding agents, including mouse DHFR (20 kDa), GFP (26 kDa), and yeast enolase (a dimer of 47 kDa subunits) (Figure 2). Surprisingly, none of these proteins were able to refold spontaneously in Ficoll 70. Instead, reactivation of these proteins was now critically dependent on the entire chaperonin system [GroEL, GroES, and ATP (Figure 2)]. After GdmCl-denatured proteins were bound to GroEL, addition of neither ATP alone nor GroES in combination with ADP or nonhydrolyzable ATP analogues led to efficient reactivation. Thus, ATP hydrolysis in combination with tight GroES binding appears to be required to release these substrate proteins and displace them into the chaperonin cavity for folding. Once the proteins had reached their folded state and were released into the crowded medium, the chaperonin system ceased to affect their functionality. This became obvious when we analyzed the effects of Ficoll 70 on the enzymatic activity of folded DHFR and the fluorescence of folded GFP. The numbers obtained in the crowding agent, which were 70 and 82% relative to the respective values in buffer, were unchanged in the presence of GroEL, GroES, and ATP (data not shown).

The observation that GroEL and ATP alone did not permit folding of DHFR, GFP, or enolase suggested the possibility that the proteins failed to dissociate from the chaperonin under these conditions in Ficoll 70. To test this possibility, ATP was added to a preformed DHFR−GroEL complex, followed by addition of GroES at later time points. This resulted in decreased refolding yields relative to those of samples where GroES was present from the beginning (Figure 3). Obviously, some of the DHFR was released, but at least a fraction of this protein was unable to rebind to GroEL, thus diminishing the size of the pool of folding-competent protein at the time of GroES addition. In the presence of trap-GroEL, yields after subsequent GroES addition were even further diminished (Figure 3). Thus, release with ATP alone is possible, but the substrate proteins apparently have difficulties in reaching their native state in the crowded bulk solution (see Figure 2) and instead cycle

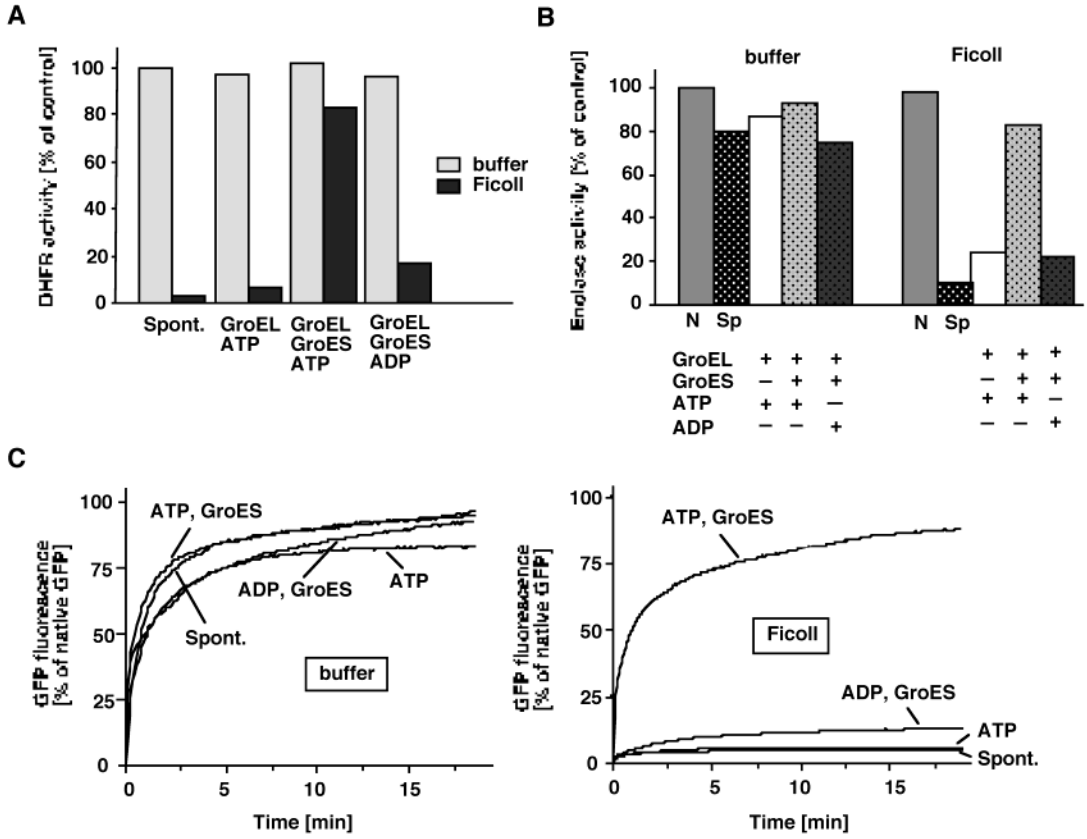

FIGURE 2: Lack of spontaneous folding and dependence on the complete GroEL/GroES chaperonin system under stringent conditions of macromolecular crowding. GdmCl-denatured proteins DHFR (A), enolase (B), and GFP (C, left and right panels) were refolded in buffer or 28% Ficoll 70 under the indicated conditions. The crowding conditions do not affect the native proteins. Enzymatic activity (enolase and DHFR) or intrinsic fluorescence (GFP) of the native proteins is set to 100%.

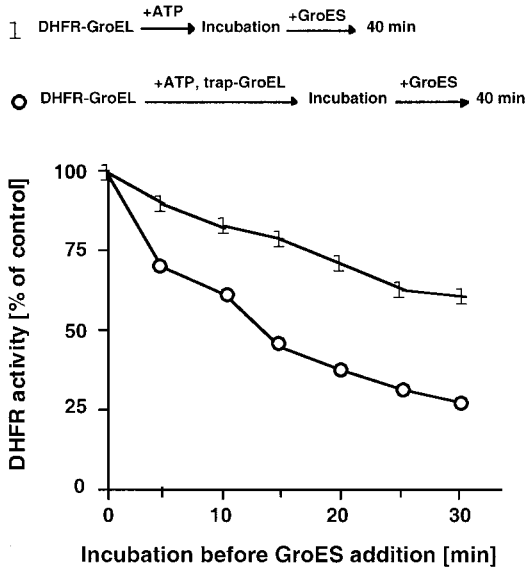

FIGURE 3: Nonproductive dissociation of substrate protein from GroEL in Ficoll 70 in the absence of GroES. DHFR−GroEL complexes in Ficoll 70 were incubated with ATP in the absence (1) or presence (○) of a 5-fold molar excess of trap-GroEL. After various times as indicated, GroES was added and refolding of DHFR was allowed to go to completion for 40 min, followed by measurement of enzymatic activity. Activity of DHFR that was refolded when GroES was present at the time of ATP addition is set to 100%.

between the GroEL-bound form and unbound non-native species. It is also possible that more non-native rhodanese is released into the crowded medium in the presence of ADP and GroES than with ATP and GroES. Crowding is known to enhance association between macromolecules, thus possibly favoring formation of smaller aggregates and rendering rhodanese incapable of rebinding to the chaperonin. However, any free non-native rhodanese should be captured early on by an excess of trap-GroEL, which has no effect on GroEL-, GroES-, and ADP-mediated reactivation of rhodanese as shown in Figure 1B. It is also possible that association between GroEL and GroES is altered in the crowding agent, thus effecting the (re)binding or substrate protein. This would not explain, however, the inability of GroEL to reactivate the typically GroES-independent substrates DHFR, enolase, and GFP with ATP alone.

What is the folding status of such non-native proteins in Ficoll 70 if they cannot rebind to GroEL or if they cannot refold spontaneously? We diluted GdmCl-denatured enolase, DHFR, and GFP into Ficoll 70 solutions and determined their sensitivity to proteinase K after 45 min of refolding time. All three proteins were protease-sensitive (Figure 4), in contrast to the situation in diluted buffer solutions, where they acquired their native conformation and became protease-resistant. Upon centrifugation, the protease-sensitive proteins in Ficoll 70 remained in the supernatant, indicating that they do not form large aggregates, but existed as non-native low-molecular weight species (Figure 4). Consistent with this finding, we did not observe any light scattering of the solutions (not shown). Nonetheless, dilution of this non-native DHFR into buffer after a previous incubation in Ficoll 70 was not effective, and folding to the native state did not resume.

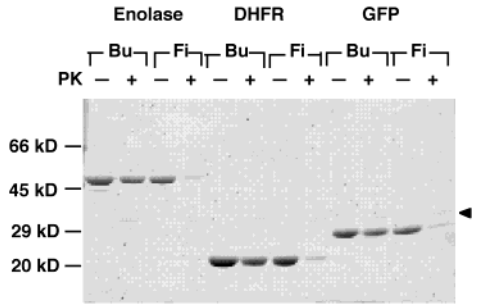

FIGURE 4: Non-native proteins accumulate in a protease-sensitive form in Ficoll 70 after dilution from a GdmCl-denatured state. Reactivation experiments were carried out with chemically denatured protein enolase, DHFR, or GFP in buffer (Bu) or in 28% Ficoll 70 (Fi). Half of each sample was subsequently treated with proteinase K (PK), followed by analysis by SDS−PAGE and Coomassie staining. The arrow marks the position of PK on the gel.

Loss of the ability to refold spontaneously was also observed recently for the reduced form of the enzyme lysozyme (*4*). However, it aggregated in Ficoll 70 upon dilution from GdmCl. This behavior is different from that of the proteins tested in the study presented here and may reflect the considerably higher concentration of lysozyme that was used compared to those of DHFR, GFP, and enolase. Moreover, reduced lysozyme already has a tendency to aggregate in typical buffer solutions (*28*), whereas DHFR, GFP, and enolase exhibit no such tendency. A common finding of both studies is that chaperones improve the folding characteristics in the crowding environment established by Ficoll 70: GroEL/GroES as described above, and protein disulfide isomerase (PDI) in the case of lysozyme (*4*). Yet another pattern of crowding effects became apparent in a recent study by Li et al. (*29*), where macromolecular crowding conditions slowed refolding rates of the enzymes PDI and glucose-6-phosphate dehydrogenase but did not reduce yields and, as in this study, did not lead to formation of large aggregates. The enhanced self-association of enzyme subunits may have favored formation of native oligomers, and also resulted in decreased refolding rates. Such positive effects on assembly would not apply to monomeric proteins such as GFP, rhodanese, or DHFR, where enhanced self-association might favor aggregation reactions instead. The inability of the proteins to refold spontaneously in the crowded solution explains their dependence on the entire system of GroEL, GroES, and ATP. Comparing the rates of folding in the presence of GroEL, GroES, and ATP shows that GFP folds in Ficoll with a $t_{1/2}$ of 33 s, whereas in buffer, $t_{1/2}$ = 20 s (Figure 2C). For enolase and DHFR, no differences in kinetics were observed (not shown). The difference in $t_{1/2}$ seen with GFP is very small compared to those observed for spontaneous refolding of lysozyme, PDI, and glucose-6-phosphate dehydrogenase in buffer versus crowding agents (*4*, *29*). Here the differences are in the range of many minutes and even hours. It may be that in the presence of GroE, parameters of the chaperonin system dominate. Binding and release of GroES, the rate of ATP hydrolysis, or the rate of substrate protein release could be rate-limiting steps and largely determine the overall folding rates of the bound substrates.

Not all nucleotides are as efficient as ATP under conditions of macromolecular crowding. The system of GroEL, GroES, and ADP, which leads to formation of ternary complexes and thus allows efficient folding in buffer solutions, is not able to provide the same assistance for the substrates DHFR, GFP, and enolase in Ficoll 70. Remarkably, however, rhodanese, which in buffer is considered the most demanding substrate of the ones tested here, is reactivated to at least some extent under these conditions in the crowding agent. One possible explanation could lie in different modes of binding to GroEL of rhodanese versus DHFR, GFP, and enolase. It has been shown that rhodanese is less stringent in the arrangement and number of contacted GroEL subunits than, for example, MDH and Rubisco (*30*). The relative strengths and types of interaction might translate into different modes of displacement and release after binding of nucleotide and GroES to the apical GroEL domains.

In summary, we have shown that certain proteins that have the ability to fold spontaneously in diluted buffer can lose this ability in a crowded environment. Under the conditions used in the experiments described herein, their folding becomes dependent on the complete GroEL/GroES chaperonin system, indicating that successful folding occurs in the chaperonin cavity. This process requires ATP hydrolysis, which suggests that the mode of initial ternary complex formation determines whether substrates can be subsequently released in a productive manner. While our data indicate that stringent conditions of crowding can impart a stronger dependence of folding proteins on the assistance by chaperonins, these results do noy imply that GFP or DHFR is strictly dependent on GroEL, GroES, and ATP in vivo. The folding conditions prevalent in the cytosol are likely to differ from the conditions employed in the in vitro experiments, as, for example, other chaperone systems (such as DnaK, DnaJ, and GrpE) are also expected to participate in the mediation of successful protein folding. While Ficoll is a recognized crowding agent, it is not expected to mimic the cellular environment in all its complexity. Indeed, refolding of proteins and chaperone activities may be affected by the nature of the crowding agents that are used (*5*, *11*, *29*). This applies also to the question of the extent to which non-native proteins are released from GroEL. While studies by Burston et al. (*11*), using concentrated *Xenopus* oocyte extracts and intact oocytes, came to the conclusion that unfolded non-native rhodanese is released in significant amounts, it was subsequently demonstrated that in intact *E. coli* cells, the quintessential setting for studying GroE, rhodanese undergoes on average only two reaction cycles at GroEL before being released in a form that is native or significantly folded (*11*, *31*).

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