Excluded Volume Effects on the Refolding and Assembly of an Oligomeric Protein

GroEL, A CASE STUDY*

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We have studied the effect of macromolecular crowding reagents, such as polysaccharides and bovine serum albumin, on the refolding of tetradecameric GroEL from urea-denatured protein monomers. The results show that productive refolding and assembly strongly depends on the presence of nucleotides (ATP or ADP) and background macromolecules. Nucleotides are required to generate an assembly-competent monomeric conformation, suggesting that proper folding of the equatorial domain of the protein subunits into a native-like structure is essential for productive assembly. Crowding modulates GroEL oligomerization in two different ways. First, it increases the tendency of refolded, monomeric GroEL to undergo self-association at equilibrium. Second, crowding can modify the relative rates of the two competing self-association reactions, namely, productive assembly into a native tetradecameric structure and unproductive aggregation. This kinetic effect is most likely exerted by modifications of the diffusion coefficient of the refolded monomers, which in turn determine the conformational properties of the interacting subunits. If they are allowed to become assembly-competent before self-association, productive oligomerization occurs; otherwise, unproductive aggregation takes place. Our data demonstrate that the spontaneous refolding and assembly of homo-oligomeric proteins, such as GroEL, can occur efficiently (70%) under crowding conditions similar to those expected in vivo.

The Escherichia coli chaperonin GroEL, a member of the highly conserved Hsp60 family, is assembled from 14 identical polypeptide chains into an oligomer with 7-fold symmetry (1–3). The oligomeric structure defines two large central cavities that have been implicated in binding nonnative protein substrates (4, 5). The apical domains of GroEL bind substrate proteins with exposed hydrophobic residues, preventing their aggregation. The substrate is displaced into the enclosed cavity after GroES binding, in an ATP-dependent reaction. Compelling evidence indicates that only the oligomeric form of chaperonin can function as a molecular chaperone (6–8). Therefore, a central question is how GroEL is folded and assembled. Several studies have described the dissociation of GroEL into monomers by chemical denaturation, proteolysis, or mutagenesis (9–13). However, data on the reconstitution of the functional chaperonin from its unfolded monomers are scarce. It has been demonstrated that monomers that have not been completely unfolded can be reassembled (9, 14, 15). Moreover, in the presence of ammonium sulfate and the Mg2+ complexes of ATP and ADP, fully denatured GroEL monomers can, to a limited extent, be reassembled into a functional, oligomeric protein (16). This finding proves that the GroEL sequence contains all of the information required for its folding and assembly, although the effect of the above mentioned reagents is not clearly understood.

Protein refolding in vitro has been extensively characterized under dilute experimental conditions to avoid aggregation of unstable intermediates. However, the intracellular environment is highly crowded due to the presence of macromolecules at concentrations such that a significant fraction of the intracellular space is not available to other macromolecular species (17–19). The so-called volume exclusion theory (17, 20) may probably be applied to protein refolding processes; indeed, there are some examples in the literature (21–24). Due to the importance of GroEL folding and assembly, we have investigated the effect of protein (BSA) and polysaccharides (Ficoll 70 and dextran 70) additives on its spontaneous refolding. Our results show that nucleotide binding, but not hydrolysis, is required to refold the urea-unfolded monomer into an assembly-competent monomer and that excluded volume conditions strongly modulate the oligomerization of the competent monomer into a tetradecameric, active protein.

EXPERIMENTAL PROCEDURES

Materials—All reagents were analytical grade. Urea, deuterium oxide (D₂O), Ficoll 70, and BSA (essentially fatty acid-free) were obtained from Sigma. Dextran 70 was obtained from Amershams Pharmacia Biotech. ¹C-Labeled urea was from Cambridge Isotope. Urea was deuterated by three consecutive lyophilizations after dilution in D₂O. GroEL was overexpressed in E. coli and purified as described (5). An additional purification step in methanol (25%, v/v) was carried out to remove most of the remaining bound substrates (25).

Refolding of GroEL—GroEL was denatured by incubating the protein 1 h in 50 mM Tris-HCl, 10 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM urea, pH 7.5, as described previously (16). Refolding

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The abbreviation used is: BSA, bovine serum albumin.

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of GroEL was initiated by a 10-fold dilution of the concentrated denatured protein into the above buffer without denaturing agent, with vigorous agitation with a Vortex mixer for 30 s, unless otherwise stated. As previously reported, this procedure results in reproducible mixing conditions (23, 26). The same results were found when the samples were diluted 15-fold (not shown). Stock solutions of crowding agents were made in refolding buffer. After the addition and mixing of all components, the final pH of the sample was 7.5 ± 0.06. In the standard assay, refolding was allowed to proceed for 30 min, at 25 °C. The results presented here are the average of at least three independent experiments with three different protein batches.

ATPase Activity Measurements—ATPase activity was measured as described previously, using a spectrophotometric method that includes an ATP-regenerating system (27, 28). Control experiments were carried out to verify that the ATPase activity of native GroEL was unaffected at 0.5 mM urea. Protein concentration as determined by the biureicnin acid assay (Sigma), was 60 nM (oligomer), and the temperature was kept at 37 ± 0.3 °C during the experiment. The effect of GroES on the ATPase activity was measured in samples containing GroEL/GroES (1:2 oligomer ratio).

Chaperonin-assisted Refolding—Refolding of rhodanase was performed as described previously (29, 30). Spontaneous folding was monitored by the recovery of rhodanase activity in the standard assay. Chaperonin-assisted refolding was measured in samples containing 170 nM GroEL, 240 nM GroES (both oligomer concentrations), and 2 mM urea on the IR spectrum of the protein (35 mg/ml), 13C-labeled chaperone. This method was used after 30 min of refolding, when the maximal yield of refolding was achieved (see “Results”), and had no effect on the process itself. However, as previously reported (16), when ammonium sulfate was present during the initial stages of refolding, the efficiency of the process was enhanced. D2O/H2O exchange was carried out to verify that the ATPase activity of native GroEL was unaffected at 37 ± 0.3 °C during the experiment. The effect of GroES on the ATPase activity was measured in samples containing GroEL/GroES (1:2 oligomer ratio).

RESULTS

Effect of Ficoll 70 on GroEL Refolding—The spontaneous refolding of GroEL from its unfolded monomers is known to be sensitive to the experimental conditions (16). The effect of Ficoll 70, a macromolecular crowding agent, on GroEL reactivation has been studied as an additional factor that might modulate its folding and assembly. The use of this “inert,” highly soluble polysaccharide was intended to mimic the intracellular crowded environment that prevails in the cytoplasm (17–20). The effect of the crowder on GroEL reactivation is shown in Table I. In the absence of nucleotides, and regardless of the presence of polysaccharide, the protein fails to refold into an active structure. In the absence of Ficoll 70, ATP slightly increases the yield of GroEL reactivation, albeit to a limited extent, in good agreement with previously published data (16). However, the simultaneous presence of 30% (w/v) Ficoll 70 and ATP in the refolding mixture induces a 3-fold enhancement in the reactivation of GroEL, as compared with the percentage obtained with only ATP. To rule out any possible effect of the background macrosolute on the ATPase activity of the protein or on the activity assay, native GroEL was incubated for 1 h with the highest concentration of crowding agents used in this study and diluted to the same extent as the refolding samples (20-fold) in the ATPase reaction mixture. The specific ATPase activity of these samples was identical to that of native GroEL (not shown), demonstrating that neither Ficoll nor the other crowders used in this study (dextran and BSA; see below) modify the ATPase activity of GroEL measured with this assay. Therefore, the observed differences in reactivation are most likely due to different refolding yields (see below).

After measuring the ATPase activity, the samples were centrifuged at 16,000 × g (4 °C, 30 min) to pellet any large aggregate that might form during refolding, and the sedimented protein was quantified (Table I). In the presence of crowder, a good correlation between the amount of protein remaining in the supernatant and the ATPase activity data is obtained, suggesting the formation of aggregates larger than the tetradecameric native protein, the latter remaining in the supernatant under these conditions. Moreover, the specific ATPase activity of the supernatant of the sample refolded in 30% Ficoll 70 and ATP was found to be similar (95%) to that of native GroEL, indicating that the nonsedimented protein has a native-like ATPase activity. In the absence of crowder, a significantly lower proportion of protein is found in the pellet than in its presence (Table I). The supernatants were submitted to a second centrifugation step (250,000 × g (4 °C, 30 min) that did not generate any visible pellet, and the soluble protein was precipitated with 5% trichloroacetic acid and quantified. In the
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Fig. 1. Original (lower traces) and deconvoluted (upper traces) infrared spectra in the amide I region of native, urea-denatured, and refolded GroEL. A, native GroEL incubated 24 h in deuterated buffer at 4 °C; B, GroEL in 5 mM 13C-labeled urea. GroEL refolded in the presence of 30% (w/v) Ficoll 70 and 5 mM ATP, in H2O (C) and D2O (D) buffer. All of the spectra were recorded in 50 mM Tris-HCl, pH 7.5 (pD = pH + 0.4), at 25 °C. Deconvolution was performed using a Lorentzian with a half-bandwidth of 18 cm⁻¹ and a band narrowing factor of 2.

presence of crowder, the results correlate well with those obtained for their corresponding pellets and ATPase activity. However, in the absence of crowder, the data suggest that refolded, inactive GroEL might not form large aggregates and might remain in solution as a monomer and/or in an intermediate oligomerization state (Ref. 16; see below). The 100% values of ATPase activity and protein concentration correspond to native GroEL incubated under the four different experimental conditions described in Table I. Similar activities were found in all four cases.

Conformational and Functional Properties of Refolded GroEL—In order to prove that the recovery of ATPase activity was due to protein refolding into a native-like structure, we compared the conformational and functional properties of refolded and native GroEL.

The secondary structure and the thermal stability of these samples were analyzed by Fourier transform infrared spectroscopy. As previously reported, the deconvoluted amide I band of native GroEL in deuterated buffer shows six components (Fig. 1A; Ref. 34), whose tentative assignment can be summarized as follows. The bands at 1656 and 1648 cm⁻¹ might represent two types of α-helices, the latter being distorted and/or solvent-exposed (35, 36). The component at 1641 cm⁻¹ contains contributions from irregular (loops, turns) and unordered structures, while the band at 1630 cm⁻¹ is characteristic of β-structures. Finally, those located at 1668 and 1681 cm⁻¹ are usually assigned to turns. The relative band areas of the components corresponding to different secondary structures, as estimated by curve fitting of the amide I band, are in reasonable agreement with the x-ray structure of the protein (not shown) and therefore support the above assignment. As expected, these signals are absent in the IR spectrum of the protein in 5 M 13C-labeled urea (Fig. 1B). It is well known that at this urea concentration, GroEL adopts an unfolded, monomeric conformation (9, 11, 14, 16). The chaotropic agent induces a downshift of the amide I band absorption maximum (from 1648 to 1641 cm⁻¹), which after deconvolution shows one major component at 1639 cm⁻¹ and a minor feature at 1672 cm⁻¹. Similar bands have been described for urea-denatured RNase (31) and are consistent with a predominantly random conformation with residual turnlike structures. The IR spectrum of GroEL refolded in 30% Ficoll and ATP, recorded after removing the crowder and the nucleotide, recovers the spectral features characteristic of native GroEL, namely the band components between 1660 and 1690 cm⁻¹ (turns); 1647 and 1657 cm⁻¹ (α-helices); and 1625 and 1630 cm⁻¹ (β-structure) (Fig. 1, C and D), suggesting that refolded GroEL adopts a native-like secondary structure. Note that the position of these component bands depends on the medium in which refolding takes place. In H2O buffer (Fig. 1C), the bands appear at higher frequencies than in D2O medium (Fig. 1D), indicating a complete exchange of the unfolded protein with the solvent when refolding is carried out in the latter buffer.

Thermal stability studies often reveal subtle differences in protein conformation. This is the case of GroEL, since it has been shown that nucleotides and the oligomerization state of the protein modulate its thermal stability (34, 37). Increasing temperatures induce clear spectral changes in the amide I absorption band of the protein (Fig. 2A). These are evidenced by the appearance of component bands at 1686 and 1618 cm⁻¹ and a broad one centered at around 1648 cm⁻¹. The former components have been related to the intermolecular aggregation of thermally unfolded protein molecules (35), and the broad band is indicative of a fluctuating conformation. The same temperature-induced spectral changes were observed for refolded monomeric and tetradecameric, active GroEL (not shown). As a consequence of these spectral changes, the amide I broadens, and the midpoint denaturation temperature (Tm) of the unfolding transition can be easily estimated by following the width of the amide I band as a function of temperature. The Tm values estimated from the “unfolding curves” shown in Fig. 2B are 73 and 72 °C for native and refolded tetradecameric GroEL, respectively. This difference is within the experimental error of the measurement. In contrast, monomeric GroEL, obtained upon refolding the protein in the absence of nucleotides and Ficoll, is remarkably less stable as demonstrated by a Tm of 53 °C. Interestingly, this Tm value is also lower than that obtained for a single ring mutant of the protein under similar experimental conditions (64 °C; Ref. 34). Unfortunately, the low solubility of monomeric GroEL hampers its conformational characterization.

The above mentioned similarity between refolded and native GroEL also extends to their functional properties, which were characterized by analyzing (i) their interaction with the cochaperonin GroES, which causes a 53 ± 4% inhibition of the ATPase activity of both samples (not shown), and (ii) their comparable ability to assist productive refolding of rhodanase (Fig. 3). As expected, refolded, monomeric GroEL is unable to assist rhodanase refolding under the same experimental conditions (Fig. 3). These data, together with the typical oligomeric structure seen by electron microscopy (Fig. 3, inset), indicate that GroEL refolds into a tetradecameric structure with conformational and functional properties similar to those of the native protein and therefore that reactivation monitors chaperonin refolding and assembly.
Requirements for Productive Refolding in the Presence of Ficoll 70—We next analyzed in more detail the effect of Ficoll 70 and different nucleotides in GroEL refolding and assembly. The time chosen to follow the refolding process (30 min) was determined by analyzing the kinetics of GroEL reactivation in the absence and presence of 30% (w/v) Ficoll 70 (Fig. 4). Although, as mentioned above, the yield of active GroEL recovery is remarkably influenced by the polysaccharide, the time dependence of the refolding reaction is similar for both experimental conditions. Assembly starts without a detectable lag period, proceeds during the first 20 min, and levels off after 30 min, regardless of the presence or absence of Ficoll 70.

The extent of GroEL refolding as a function of crowder concentration is summarized in Fig. 5. In the presence of both ATP and ADP, increasing concentrations of Ficoll 70 induce up to a 3-fold enhancement in the reactivation of the refolded protein (Fig. 5, A and B), in contrast to what is observed in their absence (Fig. 5C). This effect is clearly seen below 20% Ficoll, and higher crowder concentrations increase but slightly the reactivation yield. The concomitant increase in the ratio of tetradecameric/monomeric species observed by native electrophoresis only in the presence of nucleotides (Fig. 5, insets) further suggests that reactivation is due to protein refolding into active protein tetradecamers. It is important to mention that the ATPase activity of refolded, monomeric GroEL was always less than 8% of the activity observed for the native protein (Fig. 5C), as previously reported (7, 16). It should be noted that above 20% (w/v) Ficoll, the intensity of the bands corresponding to the monomeric and, to a lesser extent, to the tetradecameric protein species is significantly weaker (Fig. 5C, inset), suggesting that the crowder favors aggregation of the former, as described above, and might reduce the amount of native protein entering the gel due to an increased viscosity (see Table I). Considering also that the staining properties of the monomer and tetradecamer could be different (16), these results should be interpreted only qualitatively. Nevertheless, they indicate that the refolded protein is tetradecameric, with no evidence of any intermediate oligomerization state under our experimental conditions, as described previously in the absence of macromolecular crowding reagents (16). The data shown in Fig. 5 demonstrate that (i) the presence of nucleotides during the refolding process is mandatory for the protein to gain its active, oligomeric structure, and (ii) nucleotide hydrolysis is not essential for productive folding, although in the...
presence of ATP the final refolding yield is slightly higher (around 8%). Potassium, an activator of the protein ATPase activity (34, 38), enhances GroEL refolding in the absence or in the presence of Ficoll concentrations lower than 20% (Fig. 5, A and B). Increasing polysaccharide concentrations progressively diminish the potassium-induced difference in reactivation, indicating that crowding conditions attenuate the requirement of K for productive folding. This behavior applies when nucleotides are present during refolding, since no significant reactivation is observed in their absence (Fig. 5 C). Another factor that might modulate GroEL refolding is the presence of bound protein substrates in the protein preparation (15). To test this hypothesis, bound substrates were removed from GroEL by ion exchange chromatography in 25% (v/v) methanol (Fig. 5A). GroEL refolding in the presence of 5 mM ATP (A); 5 mM ADP (B); and in the absence of nucleotides (C). Final protein concentration was 1 mg/ml. Reactivation is expressed as the percentage of the ATPase activity measured for the same amount of native GroEL. Insets, native PAGE of GroEL samples refolded at the indicated Ficoll concentrations. O and M refer to the tetradesameric and monomeric protein species, respectively. WT, native GroEL that has not been unfolded.

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The requirement of nucleotides and background macromolecules for productive refolding of GroEL was further examined in two types of experiments. First, refolding was started in the presence of only one of these components (ATP or 30% Ficoll 70), and afterwards the second component was added to the refolding mixture at different times. After proper mixing and incubation of the samples (30 min, 25 °C), their ATPase activity was measured (Fig. 6A). The reactivation yield in the absence of any of these components decreases exponentially within the first minute of refolding and reaches values similar to those shown in Fig. 5A (obtained without Ficoll in the refolding mixture) and Fig. 5C (refolding in the absence of ATP). These results indicate that both components are required during the initial refolding steps to increase the reactivation yield. Second, the combined effect of protein and Ficoll concentration on GroEL refolding was characterized (Fig. 6B). In the absence of polysaccharide, increasing protein concentrations, within the experimental range measured (0.1–2.5 mg/ml), slightly and gradually enhance protein reactivation. However, when refolding takes place in the presence of different Ficoll concentrations (5, 15, and 30%), two remarkable effects are observed. First, GroEL reactivation increases with protein concentration, up to 1.5 mg/ml, in a nonlinear manner. The protein concentration at which reactivation enhancement occurs decreases with increasing Ficoll concentrations, in accordance with theoretical predictions indicating that one significant role of crowding would be to enhance self-association of the refolded, assembly-competent monomers (17). Second, when the concentration of both Ficoll (15 and 30%) and protein (above 2 and 1.5 mg/ml, respectively) become high enough, the above
mentioned polysaccharide-dependent reactivation enhancement is abolished. The protein concentration at which this behavior is observed is lower for the highest Ficoll concentration. This effect is due to protein aggregation, since when refolding is carried out in 30% Ficoll and ATP, the amount of protein found in the pellet after centrifugation (16,000 × g, 30 min) increases from 39 to 83% for GroEL concentrations of 1 and 2.5 mg/ml, respectively.

Refolding in the Presence of Dextran 70 and BSA—We next analyzed the effect of dextran 70 on GroEL refolding, as another example of “inert” crowding agent (Fig. 7). This polymer differs from Ficoll 70 in its physicochemical properties (22, 39), dextran having a more asymmetrical structure and therefore a higher viscosity than Ficoll. Both polymers increase the refolding yield of GroEL in the presence of nucleotides, Ficoll 70 being slightly more efficient. In addition to noninteracting crowders, BSA was used in an attempt to detect any specific interaction of this protein with GroEL during its refolding reaction. The results are summarized in Fig. 8. Chaperonin refolding as a function of both crowder concentration, in the presence of 5 mM ATP (Fig. 8A), and unfolded protein concentration (Fig. 8B), shows the trend described for Ficoll 70. As also noted for the polysaccharide, suppression of the beneficial effect of BSA on GroEL refolding at chaperonin concentrations higher than 2 mg/ml is due to protein aggregation. After refolding in the presence of 30% BSA and 5 mM ATP, 31 and 79% of the protein sediments (16,000 × g, 30 min) increases from 39 to 83% for GroEL concentrations of 1 and 2.5 mg/ml, respectively. However, the following differences between both crowders should be noted: (i) for the same macrosolute concentration, BSA induces a higher refolding yield than Ficoll (Fig. 8A); (ii) the increase in GroEL reactivation occurs for both polymers in a nonlinear concentration-dependent manner but at lower BSA than Ficoll concentrations (Fig. 8A); (iii) refolding in BSA saturates at 10% crowder, in contrast to what is observed for Ficoll 70 (Fig. 8A); (iv) the concentration of GroEL required to achieve maximum yields of refolding is lower in the presence of BSA (Fig. 8B); and (v) the concentration of unfolded protein above which inhibition of productive refolding is observed, is higher with BSA (Fig. 8B). These observations suggest that the excluded volume interactions exerted by these molecules of similar molecular weight might be distinct and that factors such as the different

shape of these macromolecules could also modulate the final refolding yields.

DISCUSSION

The ability of chaperonins to assemble into a functional, stable structure is an essential process that ensures productive folding of a variety of proteins required for cell viability, including GroEL itself (40, 41). In view of the biological function of GroEL, it is important to analyze the experimental requirements that allow its spontaneous folding. Up to now few (and contradictory) studies have been devoted to examine the conditions that allow refolding of monomeric, unfolded GroEL into an oligomeric, functional structure (9, 15, 16, 42). The presence of different amounts of bound protein substrates in different protein preparations (15, 42), the self-chaperoning activity of native GroEL (9), and the requirement of GroES or ammonium sulfate during the reassembly process (9, 16) have been put forward to explain the above mentioned differences. The outcome of these studies is that the spontaneous refolding of GroEL is inefficient, since unfolded, monomeric GroEL can be renatured to a limited extent only in the presence of ammonium sulfate and nucleotides (16). However, the precise role of the key element, ammonium sulfate, is not trivial to interpret, since this structure-making ion (43) might interact with the polypeptide chain during the refolding reaction (44). The study presented here takes into account a major difference between protein folding in the cell and in vitro studies: the intracellular environment is crowded due to the high concentration of macromolecules. The estimated protein concentration in the cyto-
plasm of *E. coli* (200–300 mg/ml; Refs. 45 and 46) results in a crowding effect that could increase the thermodynamic activities of macromolecules by several orders of magnitude, depending on the particular reaction (47). Crowding would, in principle, favor any state of the system that excludes the smallest volume from the highly concentrated background molecule (in the case of GroEL, a compact monomeric state (assembly-competent or not) and the oligomeric form of the protein or any other inactive aggregate).

In general, folding of an oligomeric protein, such as GroEL, is a complex process that includes most likely several pathways in kinetic and equilibrium competition (20). To simplify this otherwise extremely complex process and to qualitatively discuss our results, we shall consider the following steps: (i) adoption of a monomeric conformation competent for oligomerization and (ii) association of this conformational state into a functional oligomer.

Our data show that formation of a monomeric state competent for assembly requires the presence of nucleotides, in agreement with previously published results (16). Furthermore, these ligands must be present during the initial refolding steps, suggesting that they might interact with partially folded intermediates generated shortly after transferring the unfolded protein into the refolding buffer. Nucleotides might assist proper refolding of the equatorial domain of the GroEL subunits, which holds the nucleotide-binding site, most of the intra-ring contacts, and all of the inter-ring contacts (2). This interpretation would be in agreement with the finding that the isolated apical domain is capable of spontaneously refolding after thermal or chemical denaturation (48), in contrast to the whole protein (16, 34). The existence of a tetradecameric unfolding intermediate of GroEL with the apical and intermediate domains unfolded, together with the fact that disassembly of the native tetradecamer is closely linked to the unfolding of the equatorial domain, points to the importance of this domain in stabilizing the tetradecamer (49). Based on the latter study, on the x-ray structure of the protein (2), and on deletion mutagenesis results (12), it was proposed that the equatorial domain folds before the tetradecamer assembles, as our findings suggest. Nucleotide binding might facilitate folding of the equatorial domain by shifting the equilibrium between different folding intermediates toward a state(s) with a structure complementary to that of the ligand (50). This could also result in a stabilization of the refolded GroEL monomeric conformation, as inferred from the increased ordered structure and decreased exposure of hydrophobic surface described upon nucleotide binding to a monomeric GroEL mutant (7). Therefore, nucleotides favor the formation of one (or more) assembly-competent monomeric conformation(s) during the initial steps of refolding, that would display at the equatorial domain the appropriate protein interfaces to avoid unproductive aggregation and allow proper oligomerization. The effect of crowders on this step cannot be easily drawn from our data, since they contain kinetic contributions from both steps that, as yet, have not been separated from each other.

The second step to be considered is the association of assembly-competent monomers to reconstitute the functional homotetradecameric structure. Our experimental data reveal that the effect of excluded volume conditions on this step depends on the concentration of both the background molecule and the unfolded protein. To explain this behavior, it is necessary to take into account that proper oligomerization competes with unproductive aggregation. The effect of background molecules would be to increase the tendency of monomeric GroEL, either assembly-competent or not, to undergo self-association at equilibrium, and therefore to promote its productive oligomerization or unproductive aggregation, respectively, as experimentally observed. This interpretation is supported by excluded volume predictions (17) and by several experimental findings (22, 51, 52). However, macromolecular crowding might also change the relative rates of these competing association reactions, offering an explanation for the bell-shaped curves observed for the extent of refolding in the presence of nucleotide, as a function of GroEL concentration. The results of these experiments might be interpreted in the context of a simplified reaction scheme (Scheme 1).

In the absence of ATP, the monomeric protein species generated upon refolding are not competent for proper assembly (M*)NC, and crowding promotes its unproductive aggregation (A)n. In the presence of ATP, a more complicated picture is proposed. At low protein concentration, *i.e.* below 0.5 mg/ml, the small effect of background molecules on GroEL refolding suggests that the crowder-induced increase in the effective concentration of monomeric species is not sufficient to promote their self-association. In the 0.5–1.5 mg/ml GroEL concentration range, there is an increase in protein refolding with crowder concentration. It has been proposed that crowding slows the rate of irreversible aggregation of unfolded or partially folded protein chains (24). As the concentration of crowder increases, the rate of diffusion of partially folded conformations of GroEL decreases, and therefore refolded protein subunits would encounter each other after a period of time that might be sufficient for them to adopt a nucleotide-dependent, monomeric, assembly-competent conformation (M)C. This would lead to increased refolding of GroEL molecules into a tetradecameric, active protein particle (N)14. A further increase in GroEL concentration would shorten the encounter time, so that collision between unfolded and/or partially folded subunits (M)NC would become more probable than encounters between natively folded, assembly-competent subunits. This situation would favor aggregation versus oligomerization, and would explain suppression of the advantageous effect of crowding at high GroEL concentrations. A kinetic transformation of the assembly-competent conformation(s), (M)C, into noncompetent one(s), (M)NC2, might also be inferred from the time-dependent loss of the crowder ability to enhance refolding.

It has been suggested (53), and experimentally demonstrated for reduced lysozyme (29), that the effect of crowding on protein folding would be to greatly enhance the probability that a partially unfolded protein would aggregate. In this context, our data clearly indicate that, in contrast to what was found for lysozyme, macromolecular crowding can rescue unstable, assembly-competent GroEL monomers by promoting self-association into an oligomeric, stable protein particle with virtually identical conformational and functional properties to those of native GroEL. The underlying mechanism by which crowding has opposite effects on the reactivation of these proteins is most likely the same: the increased propensity of proteins to associate and/or aggregate in crowded media. Therefore, crowding could be beneficial in productive refolding of oligomeric proteins, such as GroEL, provided that the monomeric conformations have enough time to become assembly-competent before subunit association. It has also been pointed out that the nature of the crowding macrosolute is important in controlling
protein refolding, since besides aggregation, heterologous association is observed with some background species (23, 24, 54). The qualitative similarity of the data obtained with background macromolecules of different physicochemical properties suggests that the nature of the crowder is not critical for, although it undoubtedly modulates, GroEL refolding.

In conclusion, our findings demonstrate that crowding agents, within the range of macromolecule concentrations found in the cytoplasm of E. coli, strongly modulate the spontaneous refolding of GroEL. Productive folding critically depends on the presence of nucleotides and on the maintenance of an optimal level of total macromolecular concentration. However, it should be noted that the situation in vivo most likely includes, besides excluded volume interactions (steric repulsions), other types of nonspecific interactions (electrostatic, hydrophobic) between background molecules and any intermediate of a given protein that might influence its folding reaction, as recently pointed out (54).

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Excluded Volume Effects on the Refolding and Assembly of an Oligomeric Protein: GroEL, A CASE STUDY
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