Escherichia coli chaperonin GroEL and GroES assist in folding of a wide variety of substrate proteins in the molecular mass range of ~50 kDa, using cis mechanism, but limited information is available on how they assist in folding of larger proteins. Considering that the central cavity of GroEL can accommodate a non-native protein of ~60 kDa, it is important to study the GroEL-GroES-assisted folding of substrate proteins that are large enough for cis encapsulation. In this study, we have reported the mechanism of GroEL/GroES-assisted folding of substrates proteins that are large enough for cis encapsulation. In this study, we have reported the mechanism of GroEL/GroES-assisted folding of a 69 kDa monomeric E. coli maltodextrin glucosidase (MalZ). Coexpression of GroEL and GroES in E. coli causes a 2-fold enhancement of exogenous MalZ activity in vivo. In vitro, GroEL and GroES in the presence of ATP give rise to a 7-fold enhancement in MalZ refolding. Neither GroEL nor single ring GroEL (SR1) in the presence or absence of ATP could enhance the in vivo folding of MalZ. GroES could not encapsulate GroEL-bound MalZ. All these experimental findings suggested that GroEL/GroES-assisted folding of MalZ followed trans mechanism, whereas denatured MalZ and GroES bound to the opposite rings of a GroEL molecule. — Subhankar Paul, Chanpreet Singh, Saroj Mishra, and Tapan K. Chaudhuri1

Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, Hauz Khas, New Delhi, India

Key Words: in vivo and in vitro folding · large substrate protein · trans folding mechanism

Our understanding of the mechanism of GroEL/GroES-assisted protein folding to date has been derived mostly from studies with small substrate proteins. However, detailed information regarding the interactions between GroEL and large substrate proteins and the mode of action of GroEL in the folding process of those proteins is still poorly understood. The double ring chaperonin GroEL has been shown to assist ATP-dependent folding of many proteins (1–6) that include monomeric rhodanese (33 kDa), the subunits of malate dehydrogenase (33 kDa), and RUBISCO (50 kDa), and this has been achieved by binding of non-native proteins in an open GroEL ring through multiple hydrophobic contacts with the apical domains (7), serving to prevent misfolding and aggregation (8, 9). Two types of mechanism of action, namely cis and trans folding, have been reported for GroEL-mediated folding of substrate proteins. In the cis mechanism, an aggregation-prone folding intermediate is first captured by GroEL and thereby becomes protected from aggregation. On binding of ATP and GroES to the GroEL/polypeptide complex, the polypeptide is ejected into a closed compartment formed by the GroE chaperone, where folding is initiated. After hydrolysis of ATP, both GroES and the polypeptide are released. Since non-native protein and GroES bind to the same GroEL ring and the bound polypeptide gets encapsulated underneath GroES, this mechanism is termed as cis folding mechanism, whereas in trans mechanism, substrate protein and GroES occupy opposite rings of GroEL (10, 11). Binding of GroES and ATP to the trans ring of GroEL releases the bound polypeptide in the solution. X-ray diffraction studies on GroEL-GroES-ADP complex suggested that the nucleotide and GroES-bound GroEL central cavity can accommodate a non-native polypeptide as large as 60 kDa (12–14). In fact, to date the largest proteins that are reported to be folded using cis mechanism are a subunit of RUBISCO, a 50 kDa protein (15), and the 86 kDa αβ hetero dimer of mitochondrial branched chain α-ketoacid dehydrogenase (16, 17).

In the case of larger proteins having a molecular mass >60 kDa that are too big to be encapsulated within cis cavity of GroEL, limited information is available on the requirement of chaperonin assistance and possible mechanism for the assisted folding. However, GroEL has been found to be associated with many such proteins in vitro, and in vivo those are with a molecular...
mass >60 kDa (18–21). The non-native form of a 70 kDa tailspike protein of phage P22 binds with GroEL and is released without refolding in a GroES-independent manner (20, 21). GroEL forms a cis complex with an 86 kDa protein of 82 kDa molecular mass (yeast mitochondrial aconitase) interacts with GroEL and single ring version of GroEL (SR1) and the bound intermediate gets encapsulated by GroES and Mg-ADP (26). However, GroES could not encapsulate the GroEL-MBP-E1α fusion protein binary complex, although GroES was required for the slow recovery of E1 enzymatic activity (10). The reason for this contradiction has been explained by the anticipation (26) that in case of compact folding intermediates the size limit for cis folding is significantly >57 kDa, established for fully unfolded proteins (17, 18, 27).

Until recently, a detailed mechanistic study on GroEL-GroES-assisted in vivo and in vitro folding of a large monomeric heterologous substrate in E. coli yeast mitochondrial aconitase, demonstrated the mandatory requirement of GroEL, GroES, and ATP for its productive folding and the process follows a trans mechanism in which aconitase and GroES bind to the opposite rings of GroEL in a nucleotide-dependent way (10, 11). Further studies reveal that the bacterial cells can survive with trans folding machinery (6).

The majority of the studies on the GroEL/GroES-assisted folding of larger proteins have been attempted on the heterologous and multimeric substrates. A detailed mechanism on how GroEL and GroES assist the in vivo and in vitro folding of authentic substrates has not been demonstrated because of the scarcity of relevant substrates and difficulties in interpreting the data with the folding experiments. Furthermore, it is not quite well understood whether the mechanism of GroEL-GroES-assisted in vivo folding of heterologous proteins reflects the same scenario as that of the authentic proteins. Hence, there is a real need to understand the detailed mechanism of GroEL-GroES-assisted folding of larger (>50 kDa) substrate proteins, preferably with authentic substrates. Since the majority of the proteins within molecular mass range of 50 kDa fold through GroEL-GroES-assisted cis folding mechanism and the assisted folding process of a heterologous protein of 82 kDa molecular mass (yeast mitochondrial aconitase) follows a trans mechanism (27), there is a necessity to study the chaperone-assisted folding mechanism of proteins having molecular mass in between 50–82 kDa. This type of study can very well give rise to the true molecular mechanism of GroEL-GroES-assisted folding of large substrate proteins in E. coli cytosol.

In the present study, we have demonstrated the mechanism of GroEL-GroES-assisted in vivo and in vitro folding of E. coli maltodextrin glucosidase (MalZ), a 69 kDa monomeric protein responsible for degradation of maltodextrins to maltose by eliminating one glucose residue from the reducing end at each time (28). It has been observed that the complete chaperonin machinery is required for the production of active MalZ both in vivo and in vitro, and based on the experimental findings, we have proposed that GroEL-GroES-assisted folding of MalZ follows trans mechanism.

MATERIALS AND METHODS

Materials

DH5α and BL21 E. coli strains were used for expression and purification of MalZ, GroEL, GroES, and SR1. The pCS19MalZ containing (His)₅malZ was generous gift from Prof. W. Boos (University of Konstanz, Konstanz, Germany). The pACYCELS containing both groEL and groES genes and pACYCEL containing groEL gene, pET22dES containing groES gene and pET11aSR1 containing single ring version groEL gene were gift from Prof. A.L. Horwich (Yale University, New Haven, CT, USA). Proteinase K, 8-anilino-1-naphthalene-sulfonic acid (ANS), and p-nitrophenyl β-d-maltoside were purchased from Sigma (St. Louis, MO, USA), and the disodium salt of ATP and ADP was purchased from Sisco Research Laboratories (India). All other reagents used were of analytical grade.

Coexpression of MalZ, GroEL, and GroES

E. coli DH5α cells were transformed with pCS19MalZ plasmid, and the same cells were made competent for cotransformation with other plasmids. The DH5α-competent cells, containing pCS19MalZ plasmid, were transformed with pACYCELS or pACYCEL plasmids. In both the cases, the transformation was confirmed through checking the expression of respective proteins. Cultures of the transformed cells were grown in the LB medium (containing 80 μg/ml ampicillin or 12.5 μg/ml tetracycline for selecting pCS19MalZ or pACYCELS/pACYCEL plasmids, respectively) at 37°C and induced with 100 μM isopropyl β-D-thiogalactoside (IPTG) and left for 12–14 h for expression of malZ gene. However, groEL and groES genes were constitutively overexpressed in the cytosol. The cultures were pelleted, sonicated in the lysis buffer, and centrifuged. The supernatant was loaded on a 10% SDS-PAGE gel. Coexpression of MalZ, GroEL, and GroES was confirmed by observing intense bands on a 10% SDS-PAGE gel.

Purification of MalZ, GroEL, and GroEL mutant (D398A), SR1, and GroES

MalZ and GroEL were overexpressed in TG1 and M15 E. coli cells, respectively, and SR1, D398A, and GroES were overexpressed in BL21 E. coli cells. Plasmids pCS19MalZ, pACYCEL, pACYCELS, pET22dD398AEL, pET22dES, and pET11aSR1 were used for overexpression of MalZ, GroEL, GroEL-GroES, D398A, GroES, and SR1, respectively. Chaperones were puri-
fied as described previously (29, 30). Cells were disintegrated in French press, and lysates were centrifuged at 45,000 rpm for 45 min. The supernatant was separated and applied for chromatographic process in AKTA FPLC system. GroEL and SR1 were purified using FFQ anion-exchange chromatography, and GroES was purified using FFSP cation-exchange chromatography. MalZ purification was a single step process using Ni-chelating column HisTrap HP (Pharmacia, Piscataway, NJ, USA). After the FPLC purification process, affigel blue treatment was done on GroEL and SR1 to remove bound substrate proteins, and for GroES, differential precipitation by lowering of pH of the protein mixture caused elimination of other proteins before the FPLC purification process.

**In vivo folding of MalZ**

Monitoring of the *in vivo* folding of MalZ was based on the fact that the folded protein is soluble and physiologically active, whereas the misfolded or unfolded proteins are insoluble and have no characteristic biological properties. Hence, the amount of active soluble protein from a given amount of overexpressed protein can give rise to the extent of *in vivo* folding. *E. coli* cells containing desired recombinant plasmids were grown in the test tubes at 37°C and induced with 100 μM IPTG. After induction, all cultures were centrifuged and centrifuged and pelleted and were solubilized in lysis buffer (20 mM K HEPES, 5 mM MgCl₂, and 1 mM DTT, pH 7.4) and disintegrated by sonication at cold condition.

Cell extracts, containing same amount of total protein, were taken and centrifuged at 14000 rpm and fractionated to supernatant (soluble) and pellet (insoluble). One set of the supernatant and pellet fraction was lyophilized, solubilized in SDS-PAGE loading buffer, and loaded on a 10% SDS-PAGE gel, and the extent of *in vivo* folding was judged from the analysis of respective band intensities. Enzymatic activity of MalZ was detected from another set of supernatant fraction.

From the SDS-PAGE picture, the area of total overexpressed MalZ in supernatant fractions.

**Guandine hydrochloride induced denaturation of MalZ**

The purified MalZ (3 μM) was incubated with different concentration of GdnHCl (0–6 M) at 25°C for 4 h in 20 mM sodium phosphate buffer, pH 7.0. Denaturation of MalZ was monitored by following the loss of enzymatic activity of GdnHCl-treated samples at 37°C according to Tapio et al. (28).

**Acid-induced denaturation of MalZ**

Purified MalZ (3 μM) was denatured by 10-fold dilution with 25 mM glycine phosphate buffer, pH 2, and incubation for 4 h at 25°C. Denaturation was judged from the loss of enzymatic activity of MalZ.

**Monitoring GdnHCl induced denaturation of MalZ by intrinsic fluorescence measurement**

Intrinsic fluorescence measurements were performed using a Perkin-Elmer LS 55 spectrofluorimeter. MalZ (3.0 μM) was incubated with different concentrations of GdnHCl (0–6 M) at 25°C for 4 h in 20 mM sodium phosphate buffer, pH 7.0. Tryptophan fluorescence measurements were performed using an excitation wavelength of 279 nm and recording the emission spectra from 300 to 400 nm. Relative fluorescence intensity values were plotted against molar concentration of GdnHCl to obtain the optimum denaturant concentration for getting maximum change in fluorescence intensity.

**Determination of rate constants for the MalZ-refolding reaction**

To determine the refolding rate constant of MalZ for spontaneous and GroEL/GroES-assisted refolding, data points from percent recovery of MalZ activity vs. time plot were attempted to fit in various rate equations. However, the data points were fitted well in the first order rate equation: 

\[
\ln [A] = \ln [A]_0 - k \cdot t,
\]

where \([A] = \text{Molar concentration of the denatured MalZ at a given time; } [A]_0 = \text{Initial molar concentration of the denatured MalZ; } k = \text{Refolding rate constant of MalZ; } t = \text{Refolding time} (10).\]

RMS values corresponding to different fits were calculated, and the best-fit equation was chosen. The refolding rate constants \((k)\) and half time for refolding processes \((t_{1/2})\) were calculated from the best-fit equations.

**Proteinase K digestion experiments for determining cis encapsulation of GroEL/SR1 bound MalZ**

Denaturation of MalZ was carried out by incubating with 2 M GdnHCl for 4 h at 25°C. Denatured protein was diluted with GroEL/SR1 containing buffer such that the final concentration of MalZ and GroEL/SR1 was 1 μM. Then the GroEL-MalZ/SR1 complexes were incubated with 2 μM GroES in the presence of 5 mM ADP for 10 min to allow the formation of GroEL-MalZ-GroES-ADP/SR1-MalZ-GroES-ADP complexes. The reaction mixtures were treated with 5 μg/ml proteinase K at room temperature for 10 min, and the reaction was quenched with PMSF to a final concentration of 1 mM. To optimize the concentration of proteinase K for proteolytic digestion experiments, necessary control experiments were carried with native GroEL and MalZ. Since SR1 and GroEL exhibit the same impression in the SDS gel, a control experiment with SR1 was not performed. Digests from different reaction mixtures were then analyzed by SDS-PAGE, followed by Coomassie blue staining, to understand the extent of proteinase K protected MalZ in the GroEL-MalZ, SR1-MalZ-GroES-ADP, and GroEL-GroES-MalZ-ADP complexes.

**Identification of GroEL-MalZ and SR1-MalZ binary complexes**

MalZ was denatured with 20 mM sodium phosphate buffer, pH 7.0, containing 2 M GdnHCl at 25°C for 4 h. Denatured MalZ was diluted with 20 mM sodium phosphate buffer, pH 7.0, containing GroEL/SR1 such that the final concentration of MalZ, SR1, and GroEL was 1 μM. After 30 min of incubation at 25°C, the mixtures were centrifuged to remove any aggregated protein. Then, the reaction mixtures were run through a 24 ml gel filtration column (Superdex 75) in AKTA FPLC system (Pharmacia). The GroEL and SR1 containing fractions were collected, and aliquots from the peak top were run on a 10% SDS gel, followed by Coomassie blue staining to detect whether there was any MalZ band comigrated with GroEL/SR1.
GroEL/SR1 and GroES-assisted in vitro refolding of MalZ

MalZ was unfolded in 2 M GdnHCl or by 10-fold dilution with 25 mM glycine phosphate, pH 2.0, and incubated at 25°C for 4 h. The unfolding was confirmed by the loss of enzymatic activity of MalZ. To monitor spontaneous refolding, denatured MalZ was diluted directly into refolding buffer (20 mM sodium phosphate, pH 7.0, containing 10 mM MgCl₂, and 10 mM KCl). Chaperone-assisted refolding was carried out by diluting the denatured MalZ with the refolding buffer containing GroEL/SR1 (in such a manner that the final conc. of MalZ and GroEL/SR1 was 1 μM in the solution). After 10 min of incubation at 25°C, ATP (5 mM final conc.) and GroES (2 μM final conc.) were added to the refolding mixture. The negative control for these experiments was the buffer containing 0.022 M GdnHCl, which corresponds to the residual concentration of GdnHCl in the refolding mixture, and the positive control was the buffer containing 1 μM native MalZ protein and 0.022 M GdnHCl. MalZ activity was assayed from the refolding mixture at different time intervals up to 5 h. Percent recovery of MalZ activity in different samples was calculated after considering the equivalent amount of native MalZ activity as 100%.

To study the effect of ATP concentration on the final recovery of MalZ activity, GroEL-GroES-assisted refolding experiments were also carried out in the presence of different concentrations of ATP (5, 10, 15, 20, and 50 mM).

RESULTS

Coexpression of both GroEL and GroES significantly enhances the MalZ activity

When MalZ was overexpressed in E. coli from a lac-regulated promoter on a high copy plasmid, pCS19malZ, substantial enzymatic activity was detected in lysed cell extract (Fig. 1A, lane 2), amounting to at least 13-fold more than in an equivalent amount of cell extract from untransformed E. coli (lane 1). In the presence of constitutively overexpressed GroEL and GroES, there was 24-fold increase in MalZ activity (lane 3). When the cell extracts were analyzed by SDS-PAGE, it was observed that in the absence of exogenous GroEL/GroES, nearly 35% of the overexpressed enzyme was soluble, while the rest part had apparently misfolded and aggregated (Fig. 1B, lanes 1 and 2). In the presence of overexpressed GroEL/GroES, the soluble fraction of MalZ was increased to ~63% (lanes 3 and 4), which corresponds to the increase in enzymatic activity (data not shown). However, when MalZ was induced in the presence of overexpressed GroEL alone, the recovery of active MalZ was much less than that observed in the case of only MalZ-overexpressing cells (Fig. 1A, lane 4). Overexpressed GroEL might have prevented a large fraction of expressed MalZ from reaching to the native form. Hence, overexpression of both GroEL and GroES is necessary for the productive in vivo folding of MalZ.

Guanidinium hydrochloride deactivates MalZ

MalZ (0.3 M) was denatured by incubating with different concentration (0–6 M) of GdnHCl for 4 h at 25°C. Enzymatic activity for all GdnHCl-treated samples were measured according to the protocol reported by Tapio et al. (28). It was found that the activity of the enzyme was completely lost at 0.5 M GdnHCl (Fig. 2). To determine the limiting concentration of GdnHCl at which MalZ retains its substantial enzymatic activity, we also performed a MalZ activity test in the presence of 0–0.1 M GdnHCl concentrations (Fig. 2, inset). It was observed that MalZ activity dropped linearly in this small range of denaturant concentration. The purpose of performing this titration in the narrow range of GdnHCl was to see the activity of MalZ at 0.022 M GdnHCl, since in the present set of in vitro folding experiments all the control samples contains this much residual concentration of GdnHCl. It was observed that MalZ retains almost 90% of enzymatic activity at 0.022 M GdnHCl. Since it was observed that MalZ retains three disulphide bonds (data not shown), it was anticipated that the complete deactivation of the protein might require the reduction of disulphide bonds. However, DTT could not exhibit any additional role in the GdnHCl induced deactivation of MalZ (data not shown).
concentration of GdnHCl used for denaturation (○). Percentage of residual activity was expressed relative to activity of controls not containing GdnHCl. Changes of MalZ enzymatic activity with gradual increase of GdnHCl concentration in narrow range (0–0.1 M) is shown in inset (□).

2 (M) guanidine hydrochloride completely unfolds MalZ

MalZ (3.0 μM) was incubated with different concentration of GdnHCl (0–6 M) at 25°C for 4 h in 20 mM sodium phosphate buffer, pH 7.0. Denaturation of MalZ was monitored by measurement of intrinsic tryptophan fluorescence of the GdnHCl-treated samples. It was observed that maximum change in fluorescence intensity took place at 2 M GdnHCl (Fig. 2). The overlaid plot of change of MalZ activity and relative fluorescence intensity vs. concentration of GdnHCl demonstrated that the loss of MalZ activity preceded complete unfolding of the protein. Hence, the complete unfolding of MalZ took place at 2 M GdnHCl, as the activity was also completely lost at this denaturant concentration (Fig. 2). There is no visible change in the denaturation pattern of MalZ in the presence of 2 mM DTT (data not shown).

GroEL/SR1 form stable binary complexes with non-native MalZ

GroEL/SR1-MalZ binary complexes were formed by diluting GdnHCl denatured MalZ with the buffer containing GroEL/SR1 at 25°C and incubating the mixtures for 30 min. The final concentration of GroEL/SR1 and MalZ in the complexation reaction was 1 μM. The reaction mixtures were then run separately through a Superdex HR-75 gel filtration column in AKTA FPLC system. The peak fractions corresponding to the GroEL (Fig. 3B) and SR1 (Fig. 3D) were collected, and a small portion was loaded in a 10% SDS-PAGE gel. MalZ and GroEL protein bands were identified in the same lane, and SR1 and MalZ bands were also traced in the same lane of the SDS gel (Fig. 3E), indicating that denatured MalZ comigrates with GroEL and SR1. Hence, MalZ and GroEL/SR1 form stable binary complexes. When the other part of the purified MalZ-GroEL/MalZ-SR1 complexes were incubated with the GroES in the presence of ATP, MalZ activity was recovered from purified MalZ-GroEL binary complexes; however, no MalZ activity was recovered from SR1-MalZ complex on such treatment (data not shown). The result highlights that MalZ formed a stable binary complex with GroEL that folded productively in the presence of GroES and ATP.

Both GroEL and GroES are required for reconstitution of MalZ activity in vitro

MalZ unfolded in 2 M GdnHCl was diluted with the refolding buffer (20 mM sodium phosphate, pH 7.0, 10 mM MgCl₂, and 10 mM KCl) containing GroEL in such a manner that the final concentration of MalZ and GroEL was 1 μM in the refolding mixture and incubated for 10 min at 25°C. GroES (2 μM) and ATP (5 mM) were added to the GroEL-MalZ binary complex. For spontaneous refolding experiment, denatured MalZ was diluted with the refolding buffer only. The positive control was 1 μM MalZ in 0.022 M GdnHCl (because 2 M GdnHCl conc. was diluted 90-fold to reach 0.022 M GdnHCl conc. during refolding) with 20 mM sodium phosphate buffer pH 7.0. Refolding reaction was carried out up to 5 h after dilution (Fig. 4B). Refolding mixtures were withdrawn at different time intervals, and MalZ activity was assayed for different samples. Recovery of MalZ activity in different refolded samples was expressed as a percentage of activity achieved compared to the same amount of native protein (positive control). The spontaneous refolding of MalZ was ~7% (Fig. 4A). GroEL or GroES alone and GroEL in the presence of ATP could not enhance the extent of refolding of MalZ. However, when the refolding reaction was carried out in the presence of GroEL, GroES, and ATP, the recovery of MalZ activity reached to ~50% in 5 h (Fig. 4B), which indicated that assistance of the complete chaperone system was mandatory for the enhancement of productive folding of MalZ. There was no additional refolding over the spontaneous refolding of MalZ in the presence of SR1 alone or in the presence of SR1, GroES, and ATP, emphasizing that SR1 is not capable of folding bound MalZ even in the presence of GroES and ATP (Fig. 4A).
Multiple cycles of GroEL-mediated reactions are required for folding of MalZ

To investigate the nature of GroEL-GroES-mediated refolding process of MalZ, we decided to carry out the refolding experiments in the presence of a trap variant of GroEL-G337S/I349E (31), which can stably capture the released non-native MalZ. However, it was observed that MalZ did not bind stably enough with the GroEL trap. Hence, we used one GroEL mutant D398A (30), having identical affinities for non-native substrate and ATP similar to wild-type GroEL. This mutant hydrolyzes ATP very slowly, as a result binding of GroES and ATP to the MalZ-GroEL binary complex can promote one round of trans-driven release of GroEL-bound MalZ. The released non-native MalZ should not be able to rebind with GroEL because the open ring of a GroEL will lie opposite a ring still occupied by GroES and unhydrolyzed ATP, a state unable to bind polypeptide (32). When a folding reaction was carried out in the presence of D398A, GroES, and ATP, at early time points (30 s), ~6% of MalZ was refolded (Fig. 4C). As predicted. At later times up to 120 min, no additional activity was recovered (Fig. 4C). By comparison, in a similar reaction with a wild-type GroEL-MalZ complex (Fig. 4B), the recovery of MalZ
activity at 30 s was similar (~6%), but it increased during the subsequent minutes to 50%. Thus, we concluded that MalZ refolding involved multiple cycles of binding by GroEL and GroES-driven release in trans, with ~6% of the molecules reaching the folded state in any given round.

**GroEL/GroES-mediated refolding of acid denatured MalZ yields identical recovery of active protein as compared to the GdnHCl denatured protein**

Acid denatured MalZ was refolded by dilution with refolding buffer (20 mM sodium phosphate pH 7.0, 10 mM MgCl₂, and 10 mM KCl) containing GroEL in such a manner that the final concentration of MalZ and GroEL was 1 μM in the solution. GroES (2 μM) and ATP (5 mM) were added to the refolding mixture, and refolding was allowed to proceed for 60 min. Enzymatic assay of MalZ was performed, and the recovery of MalZ activity was found to be ~43% (Fig. 5). For the spontaneous refolding, acid denatured MalZ was diluted with refolding buffer and the extent of refolding was found to be 4.3% after 60 min. GroEL alone could not increase the recovery of active MalZ over spontaneous yield. Hence, the presence of GroEL, GroES, and ATP was mandatory for productive folding of acid denatured MalZ in vitro.

**GroEL/GroES-assisted refolding of MalZ is slower than spontaneous refolding**

The kinetic constants for the spontaneous refolding and GroEL/GroES-assisted refolding reactions of MalZ were determined from the early data points in the MalZ
activity vs. time of refolding plot (Fig. 4B). For spontaneous refolding, rate constant \( k \) was estimated to be \( 1.73 \times 10^{-3} \) s\(^{-1}\) and half time of refolding \((t_{1/2})\) was calculated to be 5.2 min, which indicated a slow refolding process. This observation was in agreement with the general consideration that the fast folding is observed only in small proteins of fewer than 100 amino acid residues (33). In the case of GroEL-GroES-mediated refolding of MalZ, the refolding rate constant \( k \) was calculated to be \( 2.98 \times 10^{-4} \) s\(^{-1}\) and half-time for refolding \( t_{1/2} = 39 \) min. The result also indicated that GroEL/GroES/ATP-mediated in vitro refolding of MalZ was slower than the spontaneous refolding process. A similar percentage of final recovery (~50%) of MalZ activity was achieved in 2 h, during GroEL-GroES-assisted refolding of GdnHCl denatured MalZ, in the presence of different concentration of ATP (10, 15, 20, and 50 mM; data not shown).

**GroES cannot encapsulate GroEL/SR1-bound MalZ**

To understand the requirement for GroES in the chaperonin-assisted refolding of MalZ, we examined whether the co-chaperonin GroES could bind to the same GroEL ring preoccupied by MalZ and encapsulate the protein in a cis ternary complex. The MalZ-GroEL/SR1 complexes were mixed with GroES in the presence of ADP, and protection of the bound polypeptide from digestion by proteinase K was assessed as a measure of GroES encapsulation. In the presence of GroES and ADP, GroEL/SR1-bound MalZ could not be traced in the gel (Fig. 6), indicating that MalZ was not protected from proteolysis by proteinase K. Hence, GroES could not encapsulate GroEL/SR1-bound MalZ. Therefore, cis ternary complex formation between GroES and MalZ-GroEL/SR1 binary complexes failed to occur.

A positive control experiment was carried out using a relatively smaller substrate protein, RUBISCO, known to be folded by GroE chaperones using cis encapsulation. Proteinase K was added to the GroEL-RUBISCO-GroES complex in the presence of ADP. It was observed that substantial amount of RUBISCO was protected from the proteolytic digestion (Supplemental Fig. 1). On the other hand, RUBISCO was completely digested when the GroEL-RUBISCO binary assembly was treated with proteinase K.

**DISCUSSION**

Recent studies on the GroEL/GroES-assisted *in vivo* folding of larger substrate proteins have been carried out on heterologous proteins in *E. coli* (11, 22, 23). Although these studies have provided preliminary information about how chaperonin GroEL/GroES interacts with the larger substrates and finally assists in their productive folding, due to difficulties in finding suitable authentic substrates amenable to the process of monitoring the interaction between the chaperonin
and substrates, no report is available till date on how larger authentic substrate proteins are folded in E. coli with the assistance of chaperonin GroEL and GroES. The mechanism of in vivo and in vitro GroEL/GroES-assisted folding of a 69 kDa monomeric E. coli protein maltodextrin glucosidase has been demonstrated here. There was a substantial increase in the in vivo folding of MalZ in the presence of exogenous GroEL and GroES. However, coexpression of GroEL alone could not exert any positive effect on the in vivo folding of MalZ. The inability of the endogenous chaperonin system to assist folding of large quantity of overexpressed MalZ might have caused the majority of the protein to be aggregated. Although there was no enhancement of in vivo folding of MalZ in the presence of exogenous GroEL, the increment in the productive folding occurred only in the presence of GroEL and GroES together. This observation clearly indicates that in vivo folding of MalZ requires assistance from the complete chaperonin machinery. Since DH5α cells were used in this study for the overexpression of MalZ, GroEL, and GroES, all these proteins are present in E. coli. Hence, the contribution of endogenous GroEL and GroES on the assisted folding of overexpressed MalZ could not be ignored. In Fig. 1A (lane 2), the extent of in vivo folding of overexpressed MalZ was probably achieved due to the action of endogenous GroEL/GroES as well as from the spontaneous folding. However, there was almost additional 2-fold increment of MalZ folding (Fig. 1A, lane 3) in the presence of exogenous GroEL/GroES compared to the previous case. Therefore, overexpression of GroEL and GroES substantially increased the in vivo folding of MalZ.

It might be noted that the expression level of MalZ was enhanced on induction of GroEL/GroES expression. Aggregation of the protein in the cytosol is often toxic in nature, which might render the synthesis of nascent proteins. When both GroEL/GroES were overexpressed in the cell, aggregation was suppressed and synthesis of recombinant MalZ was enhanced. The enhancement of MalZ production in the presence of overexpressed GroEL/GroES is not contradictory with the explanation because we have calculated the percentage of folded protein from the total overexpressed MalZ.

To understand the nature of interaction between MalZ, chaperonin GroEL/SR1, and GroES as well as the mechanistic route for the conversion of GroEL bound MalZ to the active form of the protein, in vitro studies were quite essential. GroEL formed stable binary complex with denatured MalZ, and the complex folded productively in the presence of GroES and ATP. When the denatured MalZ was diluted with the refolding buffer, a small fraction got refolded spontaneously and remaining fraction probably aggregated irreversibly. This was verified by the experimentation that the addition of GroEL/GroES and ATP a few minutes after the dilution of denatured MalZ with refolding buffer could not improve the yield of the refolded protein (data not shown). The yield of folded MalZ could not be improved by addition of GroEL and ADP in the refolding buffer. However, the substantial enhancement in the productivity of MalZ refolding occurred only in the presence of GroEL, GroES, and ATP. This information also revealed the requirement for the complete chaperonin machinery in the in vitro refolding of denatured MalZ. Hence, the agreement between the in vivo and in vitro experimental findings on the requirement of chaperonin was quite satisfactory. Furthermore, it was observed that the mode of denaturation of native MalZ could not alter the GroEL/GroES-mediated recovery of active MalZ (Figs. 4A and 5). This result demonstrated the fact that despite the different mode of denaturation of MalZ, the final denatured states were probably the same and that is why binding with GroEL and GroES-ATP-dependent release of the acid denatured MalZ yielded same extent of refolded protein as compared to the GroEL/GroES/ATP-assisted refolding of GdnHCl denatured MalZ.

The rate of spontaneous refolding of MalZ was faster (t1/2 = 5.2 min) than GroEL-GroES-assisted folding (t1/2 = 39 min). There might be several reasons for the slow refolding rate of GroEL-GroES-mediated refolding process of MalZ. Perhaps one of the possibilities was that the GroEL reaction cycles were generating inhibitory concentrations of ADP with time and the concentration of ATP was also getting reduced with time. If that was the case, the concentration of ADP in the refolding mixture exceeded to that of ATP and the efficiency of GroEL-GroES-assisted refolding of MalZ was definitely reduced. To check that possibility, GroEL-GroES-mediated refolding experiments of MalZ were carried out in the presence of higher concentrations (10, 15, 20 and 50 mM) of ATP and no enhancement of final recovery of MalZ activity was found. Hence, the relatively lower percentage of GroEL-GroES-assisted refolding of MalZ (~50%) was not due to ADP-mediated inhibition of GroEL reaction cycles or limited ATP concentration in the refolding mixture. The other potential reason for the slow rate of the GroEL-GroES-mediated refolding process of MalZ might also be due to the irreversible aggregation of nonfolded or kinetically trapped intermediates of MalZ, released after each GroEL cycle. In an independent spontaneous refolding experiment, GroEL, GroES and ATP were added after 5 and 10 min of dilution of denatured MalZ. In this case, no additional recovery of folded MalZ was achieved over the yield of spontaneous refolding (~7%). This experimental finding suggested that majority of MalZ molecules formed irreversible aggregates during spontaneous refolding process. Hence, it is also possible that during the chaperone-mediated refolding reaction of MalZ, the folding incompetent form of MalZ molecules (34, 35), released from GroEL cavity, were aggregated irreversibly so that the concentration of folding competent MalZ was reduced with time. Thus, the rate of GroEL-assisted refolding of MalZ was slower than the spontaneous folding reaction and the extent of folding could not reach 100%.

It is quite evident from the in vitro refolding reactions of MalZ that the rate of GroEL-GroES-assisted folding
of MalZ is ~6-fold slower than the spontaneous refolding reaction. To understand the reason for the slower recovery of MalZ activity in the GroEL-assisted folding of the protein, we carried out the refolding reaction in the presence of D398A mutant. Because of very slow ATPase activity of this mutant (32), binding of ATP and GroES to the MalZ-GroEL complex causes one round of trans-GroES driven release of bound MalZ molecules. Hence, the extent of refolding in each of the GroEL reaction cycle can be monitored from the D398A-mediated refolding of MalZ. The recovery of MalZ activity was ~6% at an early time point (30 s). This clearly revealed that in one GroEL reaction cycle, ~6% of the molecule was refolded. Hence, to achieve 50% of recovery of MalZ activity, several rounds of binding and releases were required. Thus, the slow rate in the chaperone-assisted refolding of MalZ might be due to the requirement of multiple reaction cycles of GroEL for the accomplishment of the optimum level of folded MalZ.

Apparently there is some confusion whether E. coli cells, generally having a doubling time of about half an hour, can survive if MalZ folds at such a slower rate in the presence of GroEL and GroES. As the rate of spontaneous folding of MalZ was quite faster, probably the small amount of spontaneously folded protein started functioning before the GroEL-GroES assistance was sought for the production of additional amount of folded MalZ. Furthermore, the rate of in vivo folding of cellular MalZ, mediated by GroEL-GroES, might be faster than that of in vitro chaperonin-assisted folding, so that the required amount of correctly folded MalZ could be generated in the cell, which might have taken care of the physiological need for the E. coli cells.

Once it was understood that GroES binding to the preformed GroEL-MalZ binary complex is essential for the productive folding of MalZ, it became obvious to understand the mode of action of GroEL and GroES on denatured MalZ leading to the correct folding. To understand whether the GroEL-bound polypeptide got encapsulated underneath GroES, a limited proteolytic digestion experiment with proteinase K was carried out on the GroEL-MalZ-ADP-GroES complex. GroEL bound MalZ could not show any resistance against the digestion. It has been reported for GroEL substrates like dihydrofolate reductase (DHFR) (36, 37) that the cis encapsulated nonnative forms of these proteins exhibited protection against proteolytic digestion. Based on these observations, it could be proposed that MalZ did not get entrapped underneath GroES.

However, it was not clear whether GroES binds to the cis GroEL ring (preoccupied by nonnative MalZ) or to the opposite ring (trans) during the chaperonin-assisted folding process of denatured MalZ. This question was answered from a different investigation in which denatured MalZ was complexed with SR1 and the complex was treated with GroES in the presence of ATP. Mutations like R452E, E461A, S463A, and V464A in the GroEL equatorial domain prevent the back to back stacking of the two GroEL rings, resulting in the formation of the single-ring complex SR1 (38). Since the second ring of tetradecameric GroEL is absent here, GroES has only the option of being bound with the SR1 ring. It was observed that neither SR1 alone nor SR1 in the presence of GroES and ATP could produce any additional amount of folded MalZ over spontaneous yield under in vitro conditions (Fig. 4A). This result demonstrated that GroES could not bind with SR1-MalZ complex because we previously demonstrated that binding of GroES to the GroEL-MalZ binary complex was essential for productive folding of MalZ. It is also understood that SR1 can fold bound DHFR and rhodanese with the concomitant binding of GroES and ATP (39, 40). In both of these cases, the GroEL bound substrates were cis encapsulated underneath GroES (36, 37, 40). Therefore, it might be suggested that GroES could not bind to the GroEL ring occupied by non-native MalZ. Thus, a trans folding mechanism can be proposed for the GroEL/GroES-assisted folding of denatured MalZ in which binding of GroES and ATP to the GroEL trans ring promotes the release of bound polypeptides in the solution as folded, folding competent, or aggregated state (Fig. 7).

**Figure 7.** Proposed model for GroEL/ES-mediated folding of a 69 kDa E. coli monomeric protein MalZ through trans mechanism where MalZ does not get cis encapsulated underneath GroES. I) Acceptor state: open ring of GroEL-GroES-ADP complex. Apical domain of the GroEL open ring is ready for binding to non-native substrate polypeptides. II) Unfolded or non-native MalZ binds to apical domain of GroEL open ring through hydrophobic interaction. III) Immediate binding of ATP to the cis-equatorial domain of GroEL triggers release of GroES and ADP from the trans ring. IV) Binding of ATP to cis GroEL ring also triggers binding of ATP and GroES to the trans GroEL ring, which immediately brings a havoc conformational change on cis GroEL ring that subsequently releases the native or folding competent MalZ intermediates into the solution. Committed form of MalZ is not perfectly folded but will become fully active while it reaches to the solution. The released intermediate of MalZ, which cannot reach to native state can rebind to GroEL and go for further cycling.
The proposed mode of action of GroES on the GroEL-bound large substrate MalZ, during the refolding, is different from the cis mechanism demonstrated for the relatively smaller proteins, within the molecular mass range of 20–50 kDa. In the cis mechanism, binding of ATP to the trans ring triggers the release of bound substrate from the cis ring (35). In the trans mechanism, binding of GroES and ATP in the trans ring is obligatory for the release of GroEL-bound substrates (11). Two obvious questions are that why E. coli cells use trans mechanism for the folding of larger substrate proteins, which are not being encapsulated underneath GroEL in the cis ring, and how folding occurs in solution. The explanation might be that the newly synthesized polypeptides, which are susceptible to misfolding, may avoid aggregation through noncovalent association with GroEL. Once the aggregation phenomenon has been avoided, the released polypeptides might reach the folded state by themselves. Unlike the cis folding mechanism with smaller substrates, MalZ folding also required multiple rounds of binding and release.

The release of MalZ was observed to be slower, which was reflected through its slow refolding reaction assisted by GroEL and GroES.

The mandatory requirement for binding of GroES in the trans GroEL ring during the chaperone-assisted folding of MalZ was investigated by carrying out refolding reaction of denatured MalZ in the presence of SR1 alone and with SR1, GroES, and ATP. Because of the absence of a second ring, GroES acts obligatory in cis complex formation on the polypeptide-bound ring.

From the present study it was observed that, wild-type GroEL-MalZ complex in the presence of GroES and ATP led to recovery of ~50% MalZ activity, whereas SR1-MalZ complex produced only ~4% recovery of MalZ activity in the presence of GroES and ATP (Fig. 4). The addition of SR1 alone to an in vitro refolding mixture could not improve the extent of refolding of MalZ diluted from denaturant, where the recovery of active MalZ was ~3.6% (Fig. 4). It was observed from the proteinase K protection study that GroES could not encapsulate GroEL-bound non-native MalZ (Fig. 6), and it was also concluded from the in vitro GroEL/GroES-assisted refolding experiments of MalZ that GroES was mandatory for its productive folding. All these experimental findings, along with SR1-, ES-, and ATP-assisted folding of MalZ, proved that MalZ and GroES must bind to the trans ring of GroEL to accomplish refolding of bound MalZ. Based on these observations, a trans folding mechanism for GroEL/GroES-assisted refolding of MalZ was proposed. We thus concluded that binding of GroES and ATP to the trans ring of GroEL (opposite to MalZ bound ring) was required for productive folding of MalZ.

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