ClpB Cooperates with DnaK, DnaJ, and GrpE in Suppressing Protein Aggregation

A NOVEL MULTI-CHAPERONE SYSTEM FROM ESCHERICHIA COLI*

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ClpB is a heat-shock protein from *Escherichia coli* with an unknown function. We studied a possible molecular chaperone activity of ClpB in vitro. Firefly luciferase was denatured in urea and then diluted into the refolding buffer (in the presence of 5 mM ATP and 0.1 mg/ml bovine serum albumin). Spontaneous reactivation of luciferase was very weak (less than 0.02% of the native activity) because of extensive aggregation. Conventional chaperone systems (GroEL/GroES and DnaK/DnaJ/GrpE) or ClpB alone did not reactivate luciferase under those conditions. However, ClpB together with DnaK/DnaJ/GrpE greatly enhanced the luciferase activity regain (up to 57% of native activity) by suppressing luciferase aggregation. This coordinated function of ClpB and DnaK/DnaJ/GrpE required ATP hydrolysis, although the ClpB ATPase was not activated by native or denatured luciferase. When the chaperones were added to the luciferase refolding solutions after 5–25 min of refolding, ClpB and DnaK/DnaJ/GrpE recovered the luciferase activity from preformed aggregates. Thus, we have identified a novel multi-chaperone system from *E. coli*, which is analogous to the Hsp104/Ssa1/Ydj1 system from yeast. ClpB is the only known bacterial Hsp100 protein capable of cooperating with other heat-shock proteins in suppressing and reversing protein aggregation.

Clp ATPases (also referred to as Hsp100 proteins) are involved in protein degradation and disaggregation in both prokaryotic and eukaryotic cells (1, 2). In *Escherichia coli*, the Clp family consists of several closely related protein-activated ATPases that associate with peptidase subunits to form ATP-dependent protease complexes. Among the identified members of the Clp family, ClpA and ClpX interact with and stimulate ClpP peptidase, whereas ClpY combines with a different peptidase, ClpQ (2). ClpB, despite a 42% sequence identity and 64% sequence similarity with ClpA (3), does not support protein degradation by Clp peptides (4).

ClpA and ClpX have chaperone-like activities in vitro, which suggests that their role in complexes with ClpP is to bind improperly folded or partially aggregated protein substrates and to deliver substrates to the peptidase. ClpX protects the bacteriophage AO protein from thermally induced aggregation (5). ClpA converts inactive dimers of RepA into active monomers (6). Unlike ClpA, ClpB does not disassemble RepA dimers (6). A yeast homolog of ClpA and ClpB, Hsp104, resolubilizes heat-induced protein aggregates in yeast cells (7). This activity requires a cooperation between Hsp104 and two other yeast heat-shock proteins: Ydj1 (Hsp40) and Ssa1 (Hsp70) (8). Interestingly, DnaK, an *E. coli* homolog of Ssa1, does not support protein disaggregation by Hsp104 and Ydj1 (8). Thus, it has not been known if protein disaggregating chaperone systems, similar to that found in yeast, exist in prokaryotes.

Among *E. coli* Clp ATPases, ClpB is the most inadequately characterized, and its possible functional role and potential protein substrates are unknown. ClpB is essential for survival of *E. coli* cells at high temperatures (9, 10), which suggests that ClpB may play a major role in the bacterial stress-response machinery. In this study, we tested whether purified ClpB has a molecular chaperone activity in vitro. We studied the refolding of firefly luciferase under the conditions of intense aggregation that could not be prevented by the conventional *E. coli* chaperones (GroEL/GroES or DnaK/DnaJ/GrpE). We found that there is a close similarity between the chaperone activities of ClpB and Hsp104. Like Hsp104, ClpB cooperates with another group of chaperones (DnaK, DnaJ, and GrpE) to suppress and reverse aggregation of a protein substrate. Thus, we have identified a novel highly efficient multi-chaperone system in *E. coli*.

While this article was in review, Motohashi et al. (11) reported identification of a chaperone system consisting of the ClpB, DnaK, DnaJ, and GrpE homologs from *Thermus thermophilus*. Those results, as well as ours, indicate that a functional cooperation between Hsp100, Hsp70, and Hsp40 families occurs not only in eukaryotes but also in prokaryotes.

EXPERIMENTAL PROCEDURES

**Proteins**—ClpB was overexpressed in *E. coli* and purified according to the procedure used to obtain ClpA (12) with small modifications (23). ClpB stock solution was prepared by dialysis against 50 mM Tris (pH 7.5), 0.2 mM KCI, 10% glycerol, 20 mM MgCl₂, 1 mM EDTA, 1 mM DTT. Concentration of ClpB was measured using the calculated absorption coefficient of A₂₈₀ = 0.38 cm²/mg (13). ClpB, like other Hsp100 proteins, forms hexamers (522 kDa) in the presence of ATP (23). Thus, concentrations throughout this paper are given for ClpB hexamers. Recombinant firefly luciferase (61 kDa) was purchased from Promega (Madison, Wisconsin). Other *E. coli* chaperones (DnaK, 69 kDa; DnaJ, 41 kDa; GrpE, 24 kDa; GroEL, 14-mer, 802 kDa; GroES, 24 kDa) were obtained from StressGen Biotechnologies (Victoria, BC, Canada). Concentrations of luciferase and chaperone stock solutions were determined by the suppliers. Bovine serum albumin was purchased from Calbiochem (La Jolla, CA), and casein was from Sigma.

**Unfolding and Refolding of Luciferase**—Luciferase (15 mg/ml) was diluted 100-fold into the denaturation buffer (30 mM Hepes, pH 7.65, 60 mM KCl, 10 mM MgCl₂, 1 mM EDTA, 10 mM DTT) containing 7 M urea and incubated for 30–45 min at room temperature.

**For refolding, the denatured luciferase was rapidly diluted 100-fold into the renaturation buffer (30 mM Hepes, pH 7.65, 120 mM KCl, 10 mM MgCl₂, 5 mM ATP, 1 mM EDTA, 10 mM DTT, 0.1 mg/ml BSA).**

Luciferase Activity Determination—Luciferase assay system was purchased from Promega. Luciferase samples were diluted with the
Chaperone Activity of ClpB

RESULTS

Firefly luciferase has been often used as a substrate in studies on chaperone function because of its tendency to aggregate after heat-shock (5, 6, 16). However, the most pronounced aggregation of luciferase occurs during refolding from the completely denatured state after chemical denaturation (8). Aggregates formed from completely unfolded proteins are a model system for the formation of inclusion bodies that occurs during refolding in vivo (17).

Fig. 1 shows the reactivation of luciferase during refolding after denaturation in urea.2 Under these experimental conditions, very low luciferase activity was recovered from the refolding reaction in the absence of chaperones (less than 0.02% of the native control, see Table I below). When ClpB or DnaK/DnaJ/GrpE were included in the refolding solution, no luciferase reactivation was observed (Fig. 1, Table I). However, when ClpB and DnaK/DnaJ/GrpE were present together in the refolding solution, a strong enhancement of the luciferase reactivation was found.

The reactivation of luciferase by ClpB and DnaK/DnaJ/GrpE was slow at room temperature, and no activity plateau was observed after more than 3 h of refolding (Fig. 1). The rate of luciferase reactivation with ClpB, DnaK, and DnaJ was lower than the rate observed when GrpE was also included (Fig. 1). There was a “lag phase” of ~40 min, during which the luciferase activity regain was very low even in the presence of ClpB and DnaK/DnaJ/GrpE. This suggests that luciferase may undergo repetitive cycles of binding to different chaperones before being released to solution in an active form.

To test whether the low efficiency of luciferase renaturation correlates with its high aggregation propensity, we evaluated the relative amounts of soluble and insoluble luciferase in the reactions with and without ClpB. After 3 h of refolding with DnaK/DnaJ/GrpE, ~60% of luciferase was in the form of large aggregates that could be removed by centrifugation (Fig. 2). However, such luciferase aggregates were not found when ClpB was included in the refolding reaction with DnaK/DnaJ/GrpE. This indicates that the chaperone-induced reactivation of luciferase, as shown in Fig. 1, is a result of suppression of the aggregation of luciferase.

Table I contains the luciferase refolding yields obtained in the presence of different sets of molecular chaperones after 160 min of refolding. The well known E. coli chaperone systems (DnaK/DnaJ/GrpE and GroEL/GroES) suppress aggregation of many protein substrates (18) are unable to increase the yield of reactivation of luciferase under the conditions used in this study (less that 0.02% of the native activity, see Table I). ClpB alone, or together with GroEL/GroES, did not increase the luciferase reactivation yield. For the significant reactivation of luciferase, it is required that ClpB is included together with DnaK and DnaJ. With 0.33 μM ClpB, 1 μM DnaK, and 1 μM DnaJ, the luciferase reactivation after 160 min increased ~10-fold, as compared with the reactivation without chaperones (Table I). Further reactivation increase (up to ~50-fold) was found when 1 μM GrpE was included together with ClpB, DnaK, and DnaJ. Thus, GrpE increases the reactivation rate but is not required for the luciferase reactivation (Table I and Fig. 1). However, both DnaK and DnaJ must be present, in addition to ClpB, to achieve luciferase reactivation. Very low luciferase activity was recovered (0.02% of the native control activity) when either DnaK or DnaJ were not included in the

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2 We found that 0.1 mM guanidinium hydrochloride inhibits ~50% of the basal ATPase activity of ClpB (not shown). However, the ClpB ATPase activity is not inhibited by urea (see Table II).
refolding solution (Table I).

Among the chaperones that reactivate luciferase, ClpB and DnaK have ATPase activity (4, 19). We found that ATP hydrolysis is required for the function of ClpB/DnaK/DnaJ/GrpE because no luciferase reactivation was observed when a nonhydrolyzable analog, ATPγS, was substituted for ATP in the refolding buffer (Table I).

The ClpB/DnaK/DnaJ/GrpE-induced luciferase reactivation strongly depended on the concentration of ClpB and DnaK/DnaJ/GrpE (Table I). The luciferase reactivation yield increased ~10-fold when the concentrations of DnaK/DnaJ/GrpE increased from 0.5 to 1 μM, and the reactivation yield increased ~36-fold when the ClpB concentration increased from 0.33 to 1.3 μM. In these studies, the concentrations of chaperones were ~10–50-fold higher than the concentration of luciferase. The chaperone concentration-dependent reactivation yields suggest that the efficiency of interchaperone interactions may be a critical limiting factor in the reactivation of luciferase by the ClpB/DnaK/DnaJ/GrpE system.

Next, we tested whether the multi-chaperone system (ClpB/DnaK/DnaJ/GrpE) is capable of rescuing luciferase from preformed aggregates. It has been observed before that luciferase could be reactivated by the yeast chaperones (Hsp104, Ssa1, Ydj1) after aggregating for 30 min at 0 °C (8). We found that large luciferase aggregates, which could be removed by centrifugation, were formed within 5 min after initialization of refolding at 0 °C without chaperones (see Fig. 3). After ~15 min of refolding, the size and/or amount of aggregates increased to the extent that some of them became irretrievable by the chaperones. However, ClpB/DnaK/DnaJ/GrpE were able to partially reanimate luciferase even after 25 min of refolding.

ClpB is a protein-activated ATPase (4), which suggests that ATP hydrolysis and the binding of a protein substrate to ClpB may be functionally coupled. We measured the rate of ATP hydrolysis by ClpB in the presence of other proteins (Table II). As observed also by previous investigators (4), the ClpB ATPase activity increased ~13-fold in the presence of casein, a highly heterogeneous and aggregated protein (20). The ClpB ATPase activation by casein was only ~4-fold when casein had been denatured in 8 M urea and then diluted into the assay buffer (Table II). No ATPase activation was observed, however, in the presence of either native or denatured luciferase. It is known that DnaJ and GrpE efficiently stimulate the ATPase activity of DnaK (19). We found that DnaJ and GrpE did not affect the ATPase activity of ClpB (Table II). These data suggest that the ClpB ATPase activity may not be stimulated during the luciferase refolding assays in Figs. 1–3.

**DISCUSSION**

Current models of the function of molecular chaperones are focused on their ability to prevent aggregation of protein substrates by binding to partially folded aggregation-prone protein conformations (18). Although some chaperones can rescue misfolded proteins from early reversible pre-aggregation stages, they are unable to reverse protein aggregation after it occurs (21). Yeast chaperones, Hsp104, Ssa1, and Ydj1, have been the first discovered example of a cellular machinery capable of resolubilizing aggregated proteins and recovering protein activity from large aggregates (8). It is also an example of functional cooperation between chaperones from different heat-shock families.
Our studies identified ClpB as a member of a highly efficient molecular chaperone system in *E. coli*. ClpB, together with DnaK, DnaJ, and GrpE, suppresses aggregation of luciferase (Figs. 1 and 2). At this time, we do not know if the above chaperones bind partially folded conformations of luciferase before it aggregates. However, our data indicate that the ClpB/DnaK/DnaJ/GrpE system is capable of reactivating luciferase after severe aggregation (Fig. 3). Because Clp ATPases and DnaJ are unstable in low ionic-strength buffers (12, 22), we used 0.12 M KCl in the luciferase refolding solution, which drastically increased aggregation of luciferase and decreased the spontaneous recovery of the luciferase activity (see Table I), as compared with the refolding conditions used in previous studies (8). Neither GroEL/GroES nor DnaK/DnaJ/GrpE chaperone systems could prevent aggregation of luciferase under these conditions (Table I). However, the ClpB/DnaK/DnaJ/GrpE system increased the luciferase reactivation yield up to ~3,000-fold (Table I).

The present studies extend the pioneer work of Lindquist and co-workers (1, 7, 8) on the function of Hsp104 in yeast into prokaryotic stress-response systems and confirm that Hsp100 proteins may be the most potent aggregation suppressants known to date. Also, our studies show previously unrecognized capabilities of the well studied *E. coli* chaperones: DnaK, DnaJ, and GrpE. It has been found before that, under the conditions of less severe aggregation of luciferase than those employed in this study, DnaJ binds denatured luciferase and targets it to DnaK, which prevents luciferase aggregation (16). GrpE participates in dissociating the luciferase-DnaK/DnaJ complex. We do not know, at present, which of these chaperones interact with ClpB and how the luciferase substrate is shuttled between ClpB and DnaK/DnaJ/GrpE. To understand the mechanism of refolding reactions described in this study, it will be essential to characterize interactions of ClpB with DnaK/DnaJ/GrpE and to identify the sequence of substrate binding and substrate remodeling steps taking place in this chaperone system.

ClpB may not be the only Clp ATPase capable of cooperating with other *E. coli* chaperones in suppressing protein aggregation. However, although ClpA protects luciferase from heat-inactivation, it does not affect the yield of luciferase reactivation by DnaK and DnaJ (6). Because ClpB has not been implicated in protein degradation processes in *E. coli*, overexpression of ClpB, possibly together with DnaK/DnaJ/GrpE, may be potentially useful in preventing protein aggregation and improving recovery of recombinant proteins.

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