Hsp70 Chaperone Machine Remodels Protein Aggregates at the Initial Step of Hsp70-Hsp100-dependent Disaggregation∗

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Exposure to temperatures over a certain limit leads to massive protein aggregation in the cell. Disaggregation of such aggregates is largely dependent on the Hsp100 and Hsp70 chaperones. The exact role of the Hsp70 chaperone machine (composed of DnaK, DnaJ, and GrpE) in the Hsp100-dependent process remains unknown. In this study we focused on the Hsp70 role at the initial step of the disaggregation process. Two different aggregated model substrates, green fluorescent protein (GFP) and firefly luciferase, were incubated with the Hsp70 machine resulting in efficient fragmentation of large aggregates into smaller ones. Our data suggest that the observed fragmentation is achieved first by extraction of polypeptides from aggregates in Hsp70 chaperone machine-dependent manner and not by direct fragmentation of large aggregates. In the absence of Hsp100 (ClpB) these “extracted” polypeptides were not able to fold properly and promptly reassocitated into new aggregates. The extracted GFP molecules were efficiently recognized and sequestered by a molecular trap, the mutant GroEL D87K, which binds stably to unfolded but not to native polypeptides. The binding of extracted GFP molecules to the GroEL trap prevented their reaggregation. We propose that the Hsp70 machine disentangles polypeptides from protein aggregates prior to Hsp100 action.

The chaperone protein network controls proper protein folding and maintenance in the cell (1–3). Increases in temperature above the physiological level result in massive protein aggregation in the cell (4–6). Survival under such severe conditions is enhanced by preconditioning the cells at sublethal temperatures. This phenomenon of acquired thermotolerance depends on the presence of the AAA+ (ATPase associated with diverse cellular activities) chaperones, e.g. ClpB in Escherichia coli (7, 8) and Hsp104 in the yeast Saccharomyces cerevisiae (9, 10). AAA+ proteins self-assemble into oligomeric structures and use the energy from ATP hydrolysis to remodel their target substrates (11). ClpB belongs to the Hsp100 family and consists of an N-terminal domain and two ATP binding domains, which are essential for ClpB hexamerization and chaperone function (12–16). The first AAA+ domain contains an additional coiled-coil region called “middle domain” located C-terminally to ATP binding site that differentiates ClpB from other E. coli Hsp100 family members (17). In contrast to ClpB the other E. coli Hsp100s function as protease regulatory subunits and translocate the substrates to be proteolyzed through their central channels (18, 19).

Electron microscopic studies revealed that the protein aggregates formed under severe heat shock conditions are not eliminated in clpB or hsp104 mutant cells, compared with wild type strains (4–6). On the other hand, in vitro studies on the reactivation of aggregated proteins showed that ClpB or Hsp104 chaperones alone are not sufficient for disaggregation of aggregated substrates. The simultaneous presence of Hsp100 and co-chaperones is required for efficient substrate disaggregation and reactivation (20–24). Hsp100 (DnaK in E. coli) and its co-chaperones (DnaJ and GrpE) are involved in several cellular processes including initial folding accompanying protein synthesis (2, 3), translocation through membranes (25), remodeling of protein complexes (26), regulation of the heat shock response (27), and control of protein aggregation (20–24, 28).

The mechanism of Hsp100-Hsp70-dependent disaggregation of aggregated substrates is not fully understood yet. The requirement of ClpB protein in reactivation depends on aggregate properties and not necessarily on protein sequence. For some proteins (for example, glucose-6-phosphate dehydrogenase) varying denaturation conditions may lead to obtaining aggregates that are efficiently reactivated by Hsp100 system alone, or aggregates that require the presence of Hsp100 (29). Although those aggregates differ in size, it is not clear whether this is a factor determining ClpB requirement, or whether other physico-chemical properties are involved. This ambiguity in ClpB requirement led initially to preliminary models that postulated interaction with ClpB as initial step that render ClpB-requiring aggregates into DnaK/DnaJ/GrpE-recognizable form (30). Only recently have our kinetic studies (31) and the elegant work from Bukau and co-workers (32) pointed to the role of Hsp70 at the initial stage of the disaggregation reaction. Additionally, the studies of the Bukau and co-workers (32) determined that polypeptides are translocated through the 16 Å axial channel present in the ClpB hexameric structure (33). The importance of integrity of the channel for the disaggregating activity of ClpB was shown by mutagenesis studies (34, 35).

Although substantial progress has recently been made in understanding the functioning of the Hsp100/Hsp70 chaperone machine, several questions remain open as pointed out by Shorter and Lindquist (36). For example, the role of the Hsp70 machine at the initial step of disaggregation reaction is unknown. One of the possible mechanisms assumes that the Hsp70 chaperone system is involved in proper positioning of ClpB at aggregates. Then the properly positioned ClpB uses its “crowbar” activity for remodeling of aggregates. The large coiled-coil middle domain of ClpB, characteristic for a subfamily of Hsp100 chaperones, was postulated to be important for such ClpB activity, because the mutagenesis studies (33) determined that the mobility of this domain is critical for ClpB-dependent disaggregation. In another scenario the Hsp70 machine performs a more active function at the initial step of disaggregation (31).

In this study we focus our attention into the role of the Hsp70 machine in these initial steps of protein disaggregation. To do this, two different substrates, whose disaggregation is dependent on the simulta-
neous presence of both the Hsp70 and Hsp100 chaperones, were incubated with the Hsp70 chaperone machine. In both cases this resulted in efficient fragmentation of large aggregates into much smaller ones. Taken together, our data suggest that the observed fragmentation of aggregates is achieved through the Hsp70-dependent extraction of polypeptides from aggregates. In the absence of ClpB these polypeptides are not able to fold properly and reassociate into new aggregates. We postulate that the Hsp70 machine disentangles polypeptides from protein aggregates prior to Hsp100 action.

MATERIALS AND METHODS

Protein Purification and Concentrations—Published protocols were used for the purification of E. coli DnaK, DnaJ, GrpE (37), ClpB (38), GroEL, and GroEL D87K (39). GFP2 was purified and heat aggregated as described previously (31). Recombinant firefly luciferase was purchased from Promega. Protein concentrations were determined with the Bio-Rad protein assay using bovine serum albumin as a standard. Molar concentrations are given assuming a hexameric structure for ClpB, 14-mer for GroEL, heptameric for GroEL D87K, and a monomeric structure for the rest of proteins.

Sedimentation Analysis—Experiments were performed essentially as previously described (27). Reaction mixtures (100 l) contained native or heat-aggregated GFP (2.2 mM) and chaperones, 4.4 mM DnaK, 0.34 mM DnaJ, 0.45 mM GrpE, and 3.3 mM ClpB as indicated, in buffer A (40 mM Tris-HCl (pH 7.8), 85 mM potassium glutamate, 20 mM MgCl2, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol, 5 mM ATP or other indicated nucleotide), and were assembled on ice. After a 15-min incubation at 25 °C the reaction mixtures were applied on a 3.5-ml 15–45% (v/v) glycerol gradient in 40 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM MgCl2, 5 mM 2-mercaptoethanol buffer containing 1 mM nucleotides, where indicated. Gradients were centrifuged at 4 °C in a Beckman SW 60 rotor at 40,000 rpm for 2 h; fractions were collected from the top. Proteins present in fractions and at the bottom of the centrifugation tube were separated by SDS-PAGE. GFP was visualized by Western blot using anti-GFP monoclonal mouse antibodies (Roche Applied Science) and a chemiluminescent detection system (SuperSignal West Pico, Pierce). Polyclonal rabbit antibodies (Sigma) were used for luciferase. Luciferase aggregates were obtained by incubation of 60 mM luciferase for 60 min at 30 °C in 2 mM guanidine-HCl in 40 mM Tris-HCl (pH 7.4), 50 mM KCl, 1 mM dithiothreitol, 15 mM Mg-aceacetate and subsequent dilution on ice into buffer without denaturant. Native or aggregated luciferase (1 mM) was used for reaction with chaperones and sedimentation.

In the experiments presented in Figs. 3–5, gradients were centrifuged for 16 h. Reaction mixtures in Figs. 4 and 5 were incubated for 5 min with chaperone proteins (KJEB and KIE, respectively) before GroEL D87K (5.5 mM) was added. Incubation proceeded for another 40 min before the reactions were sedimented.

Sizing Chromatography Experiments—Reaction mixtures (100 l) containing GFP and chaperones were assembled as for sedimentation experiments and loaded onto a Sephacryl S-500 HR column (20 × 0.7 cm) equilibrated with buffer A. Chromatography was carried out at a flow rate of 0.1 ml/min at 4 °C. Fractions (400 l) were collected and analyzed by SDS-PAGE followed by Western blot analysis for GFP. The column was characterized by sizing intact E. coli cells overproducing GFP for void volume determination, acetone for the total volume of the column, and dextran blue as the 2-MDa standard.

Spontaneous Folding of Acid-denatured GFP—Twenty ml of acid-denatured GFP (in 10 mM HCl, 1 mM dithiothreitol, 0.3 mM EDTA) were added to a cuvette (GFP at 25 nm final concentration) containing 400 ml of buffer B (40 mM Tris-HCl (pH 7.8), 50 mM NaCl, 10 mM MgCl2, 10% (v/v) glycerol) and, where indicated, GroEL or GroEL-trap (125 nm). After 2 min ATP was added to 10 mM concentration. Spontaneous folding of GFP, manifested by an increase in fluorescence, was measured in a PerkinElmer LS50B spectrophotometer (excitation at 395 nm, emission recorded at 510 nm, 5.5 nm excitation and 10 nm emission slits). The fluorescence was proportional to the native GFP concentration.

GFP Disaggregation Experiments—Experiments were performed as described previously (31). Briefly, disaggregation reactions were performed at 25 °C in a spectrofluorometric cuvette (final volume 450 l) in buffer A with 5 mM ATP. The reactivation was started by the addition of thermally denatured GFP (0.5 mM) to the cuvette containing 1 mM DnaK, 0.2 mM DnaJ, 0.1 mM GrpE, 0.65 mM ClpB and, where indicated, GroEL (2.5 mM) and GroE (2.5 mM). GroEL D87K ("GroEL-trap," 2.5 mM) was added after 5 min of reactivation by KJEB. GFP fluorescence was recorded as described above.

Light Scattering Experiments—Light scattering experiments were essentially performed as described in Ref. 31. Light scattering was measured in a PerkinElmer LS50B spectrophotometer with the excitation and emission set at 550 nm. The entrance and emission slits were set to 5 nm. The solution of chaperones was subjected to a short spin prior to addition to cuvette.

RESULTS

Influence of the Hsp70 Chaperone Machine on the Macroscopic Properties of Aggregates—Recent reports on kinetic (31) and mechanistic (32) aspects of protein disaggregation placed the Hsp70 chaperone machine at a step prior to Hsp100 action during the initial stages of the reaction. We decided to investigate in detail the role of the Hsp70 chaperone machine (DnaK, DnaJ, and GrpE; referred to as K/J/E) during these initial steps. We selected GFP as our model substrate because heat-induced aggregates require both K/J/E and ClpB chaperones for efficient disaggregation and reactivation (31). We started by first investigating the influence of the K/J/E chaperone machine on macroscopic properties of GFP aggregates. The sedimentation analysis conditions (15–45% glycerol gradient, 40,000 rpm, Beckman SW 60 rotor, 2 h) were chosen in such a way that soluble proteins barely enter the gradient because of their relatively low sedimentation coefficient and the short time of sedimentation. Conversely, these conditions allow separation of much larger structures such as protein aggregates. Consequently, the majority of heat-induced GFP aggregates reached the bottom of the tube while the rest were found in fractions close to the bottom (Fig. 1A). However, when GFP aggregates are incubated with K/J/E in the presence of ATP prior to sedimentation, their distribution pattern changes strikingly and nearly all GFP is found in fractions 4–9 (Fig. 1A) close to the top of the gradient. In contrast, incubation of aggregated GFP with ClpB alone does not change the sedimentation pattern (result not shown). Native GFP sediments at the top of the gradient under these conditions (fraction 1) (Fig. 1A), whereas the bacterial RNA polymerase, a multisubunit protein (β, β′, 2α; Mw ~ 400 kDa) is found in fraction 3.

To extend our analysis to other substrates, a similar sedimentation was performed for firefly luciferase. Luciferase aggregates were obtained by denaturation in guanidine hydrochloride and dilution into the buffer without denaturant. Similarly to GFP, luciferase disaggregation depends on the presence of both K/J/E and ClpB. Following sedimentation, luciferase aggregates are found at the bottom of the tube (Fig. 1B). Incuba-
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Conversion of Aggregates Relies on the ATP-dependent Action of the Complete Hsp70 Chaperone Machine—We next asked whether all three chaperones of the Hsp70 machine, namely DnaK, DnaJ, and GrpE, are required for the observed transformation of aggregates. Heat-induced GFP aggregates, incubated in the presence of ATP and different combinations of chaperones (K/E or K/J or J/E), were sedimented as described above, and the pellet at the bottom of the gradient tube was analyzed. Only when all three K/J/E chaperones were present during incubation did the GFP aggregates efficiently fragment and were therefore missing from the bottom fraction (Fig. 2A). Omission of either DnaK or DnaJ blocked the fragmentation of aggregates (Fig. 2A). However, when GrpE was omitted from the reaction mixture, the fragmentation of GFP aggregates was observed, yet was less efficient (Fig. 2A). Replacement of DnaJ by the mutant DnaJ259 also prevented the fragmentation of aggregates (Fig. 2A). The DnaJ259 protein possesses an amino acid substitution (a conserved histidine to a glutamine) within the strictly conserved so-called “J domain.” The mutant is defective in stimulation of the ATPase activity of DnaK but not in interaction with substrate proteins (40, 41).

We also analyzed nucleotide requirements of fragmentation reaction. GFP aggregates were incubated in the presence of K/J/E and different nucleotides (ATP, ADP, ATPγS) then subjected to sedimentation. Efficient fragmentation of GFP aggregates, manifested by the lack of GFP in the pellet at the bottom of centrifugation tube, was observed only when ATP was present during incubation of aggregates with the Hsp70 machine (Fig. 2B). Replacement of ATP by ADP or ATPγS or no nucleotide did not result in fragmentation. These results indicated that the fragmentation of large aggregates into smaller ones is consistent with the cyclic interaction of DnaK with the substrate protein driven by the ATPase cycle and coordinated by co-chaperones DnaJ and GrpE.

We previously used light scattering measurements to analyze the kinetics of GFP disaggregation reaction by ClpB-K/J/E bichaperone system (31). We decided to apply this technique to analyze the K/J/E-dependent fragmentation of GFP aggregates. Heat-aggregated GFP was placed in a spectrofluorometric cuvette, and the change in light scattering was monitored following addition of K/J/E chaperones. When K/J/E were added simultaneously to the cuvette containing aggregated GFP and ATP, the light scattering signal increased to reach the maximum in ~1 min (Fig. 2C). Then, the order of protein addition was changed, and the K/E chaperones and ATP were added to the cuvette first, and the reaction was initiated by addition of aggregated GFP (Fig. 2C). The observed changes in light scattering signal were virtually identical to those recorded previously. We analyzed also the requirement for nucleotides in this reaction. Simultaneous addition of aggregated GFP and K/J/E to the cuvette did not result in increase of light scattering unless ATP was added (Fig. 2C). The replacement of ATP with ADP or ATPγS did not influence the signal (result not shown). From these experiments we concluded that the observed increase of light scattering reflects the change in the nature of aggregates in response to the ATP-dependent action of K/J/E chaperones. The light scattering (Fig. 2C) and sedimentation experiments (Fig. 2, A and B) gave corresponding results. A characteristic fast increase of light scattering signal took place only in conditions in which large GFP aggregates were transformed into smaller ones.

The GroEL D87K Trap Mutant as a Molecular Tool to Study the Mechanism of GFP Aggregates Conversion—What is the biochemical mechanism of the transition from “large” to “small” aggregates? The formation of small aggregates may be a result of direct fragmentation of large aggregates by the K/J/E chaperone machine through a new unknown mechanism. Alternatively and more likely, the K/J/E chaper-
ones may disentangle GFP polypeptides from large aggregates, followed by reassociation of these polypeptides into new smaller aggregates because of their inability to fold properly in the absence of ClpB. If the second prediction is correct, it would suggest that the Hsp70 machine is involved in the remodeling of aggregates leading to their dissociation at the beginning of the reaction. The description of this process is of major importance for the proper assignment of a role to the Hsp70 chaperone machine during the initial steps of the Hsp70-Hsp100-dependent disaggregation reaction. To address this problem experimentally, we needed to distinguish between the aggregates composed of many polypeptides and single unfolded polypeptides, which are expected to be present for a relatively short time. Therefore, we took advantage of the properties of a specific “trap” mutant of the GroEL chaperonin.

The GroEL D87K mutant is known to bind stably to unfolded polypeptide chains (19, 39, 42). Therefore, we analyzed the binding of this GroEL-trap to unfolded GFP. GFP was denatured by incubation at low pH. Dilution into neutral pH buffer results in spontaneous folding of GFP (Fig. 3). In the absence of ATP, the addition of both wild type GroEL and GroEL-trap blocks folding. Following ATP addition, GFP is released from wild type GroEL, allowing refolding, but not from GroEL-trap (Fig. 3). We performed additional sedimentation experiments to analyze the protein complexes formed during the inhibition of GFP folding by GroEL-trap. In these experiments the time of sedimentation was increased to 16 h (versus 2 h in the experiments presented in Figs. 1 and 2). The longer sedimentation time allows better separation of monomeric proteins and their complexes, however, at the cost of being unable to differentiate between the large and small aggregates because both types are large enough to sediment at the bottom of the tube under these experimental conditions. When acid-denatured GFP was diluted into buffer containing a 2.5-molar excess of GroEL-trap and loaded on the gradient, a substantial portion co-sedimented with heptameric GroEL-trap (Fig. 3). The rest either sedimented at the position characteristic for native monomeric GFP or formed a pellet, suggesting that a small fraction of the GFP aggregated. The GFP/GroEL-trap complex sedimented to the same position in the gradient as heptameric GroEL-trap alone, suggesting that monomers of GFP associate with heptameric GroEL (Fig. 3 and result not shown). Moreover, no GroEL-trap was found at the bottom of the tube in the fraction containing aggregated GFP, strongly suggesting that GroEL-trap does not interact with GFP aggregates (Fig. 3). In a control experiment we showed that GroEL-trap did not form a complex with native GFP (Fig. 3). From this set of experiments we concluded that in the presence of ATP GroEL-trap stably binds unfolded GFP but neither native GFP nor GFP aggregates.

The Hsp70 Chaperone Machine Extracts the GFP Polypeptides from Large Aggregates—Having characterized the ability of GroEL-trap to bind different forms of GFP, we used it as a tool to address the question of the mechanism of substrate disaggregation by the Hsp70-Hsp100 chaperone machines. First, we assessed how the ability of GroEL-trap to bind unfolded polypeptides influences K/J/E-ClpB-dependent disaggregation of heat-denatured GFP. The rate of GFP disaggregation and refolding was followed by spectrofluorometric measurements as previously described (31). Addition of GroEL-trap to the reaction containing heat-aggregated GFP and K/J/E-ClpB efficiently inhibits the GFP refolding (Fig. 4A). In a control experiment we showed that replacement of GroEL-trap mutant by the same amount of wild type GroEL did not influence either the folding rate or the yield of reactivated GFP (Fig. 4A). Sedimentation analysis (the 16-h scheme) of the products of a GroEL-trap-inhibited disaggregation reaction revealed that a complex between GroEL-trap and GFP is formed (Fig. 4B). This result supports the idea that unfolded GFP polypeptides recognizable by GroEL-trap do appear during the disaggregation reaction. These polypeptides most likely form during the initial K/J/E-dependent step of disaggregation, as suggested by our experiments showing the large to small aggregates transition (Figs. 1 and 2), or alternatively the trap recognizes...
Acid-denatured GFP (increase in the fluorescence of acid-denatured GFP during its spontaneous refolding. Native or acid-denatured GFP (2.2 M) was added to the cuvette to release the GFP bound by chaperonins. GroEL-trap binds acid-denatured but not native GFP. Native or acid-denatured GFP (2.2 M) was added to neutral pH buffer containing GroEL-trap (11 µM) and ATP (10 mM). The reactions were subjected to the 16-h sedimentation. GFP co-sedimented with GroEL-trap in the middle of the gradient (Fig. 5), in the same fractions as GroEL-trap (11 µM) and ATP (10 mM). The reactions were subjected to sedimentation in a glycerol gradient for 16 h. The last panel shows the control reaction in which acid-denatured GFP was allowed to fold in neutral pH buffer in the absence of GroEL-trap. Fractions were analyzed by SDS-PAGE and Western blot for GFP and Coomassie Brilliant Blue staining for GroEL-trap.

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FIGURE 3. The GroEL-trap binds stably to unfolded GFP and inhibits its folding. A, increase in the fluorescence of acid-denatured GFP during its spontaneous refolding. Acid-denatured GFP (GFPAD) was added to the neutral pH buffer with or without GroEL-trap or wild type GroEL, and GFP fluorescence was monitored. ATP (10 mM) was added to the cuvette to release the GFP bound by chaperonins. B, GroEL-trap binds acid-denatured but not native GFP. Native or acid-denatured GFP (2.2 M) was added to neutral pH buffer containing GroEL-trap (11 µM) and ATP (10 mM). The reactions were subjected to sedimentation in a glycerol gradient for 16 h. The last panel shows the control reaction in which acid-denatured GFP was allowed to fold in neutral pH buffer in the absence of GroEL-trap. Fractions were analyzed by SDS-PAGE and Western blot for GFP and Coomassie Brilliant Blue staining for GroEL-trap.

FIGURE 4. GroEL-trap inhibits K/J/E-ClpB-dependent disaggregation of thermally aggregated GFP by binding to GFP polypeptides. A, increase in GFP fluorescence during disaggregation is inhibited by GroEL-trap. The reactivation was initiated by the addition of thermally aggregated GFP to the cuvette containing DnaK, DnaJ, GrpE, ClpB alone (trace KJEB) or together with GroEL and GroE (KJEB/GroELS). GroEL-trap was added after 5 min of reactivation by KJEB. B, 16-h sedimentation of protein complexes formed after addition of GroEL-trap to the reaction of GFP disaggregation by DnaK, DnaJ, GrpE, and ClpB. Fractions were analyzed by SDS-PAGE followed by Western blot for GFP (top) and Coomassie Brilliant Blue staining for other proteins (bottom). GroEL-trap present in fractions 12–15 and ClpB in fractions 5 and 6.

FIGURE 5. GFP polypeptides are recognized by GroEL-trap as a result of K/J/E-dependent remodeling of aggregates. Thermally aggregated GFP was incubated in the presence or absence of DnaK, DnaJ, and GrpE chaperones for 5 min. Following this, the reactions were divided, and to one part GroEL-trap was added. After incubation for another 40 min reactions were sedimented for 16 h. Fractions were analyzed by SDS-PAGE and Western blot for GFP.

In a control experiment we showed that only very small amounts of GFP are complexed with GroEL-trap when the K/J/E chaperones are omitted from the reaction, and aggregated GFP is incubated and sedimented with GroEL-trap only (Fig. 5). When GroEL-trap is omitted from the reaction, both large aggregates (without K/J/E) and small aggregates formed in the presence of the K/J/E chaperones are found nearly exclusively at the bottom, just as expected for the 16-h sedimentation (Fig. 5).

The ability of GroEL-trap to bind to unfolded GFP polypeptides during the K/J/E dependent transformation of large to small aggregates was additionally investigated in light scattering experiments. This transformation is manifested by increase of light scattering signal in response to the addition of K/J/E chaperones and ATP to GFP aggregates (Figs. 2 and 6A). The presence of GroEL-trap in the cuvette substantially inhibited the characteristic increase of light scattering signal in response to addition of K/J/E chaperones (Fig. 6A). This suggests that the small
This places the role of ClpB at the later step of the reaction. The pres-
ence of ClpB prevents the liberated polypeptides from reaggregating.
The role postulated by us for ClpB does not necessarily contradict the
finding that this chaperone cannot prevent protein aggregation in vivo
(5). The isolation of polypeptides dissociated from aggregates is a spe-
cialized process requiring K/J/E chaperones and may not be compatible
with the unfolding and aggregation states present during thermal dena-
turation of proteins.

The presence of unfolded GFP polypeptides following coordinated
K/J/E chaperone action was strongly suggested by our experiments in
which a specific molecular trap, the GroEL (D87K) chaperonin mutant,
which stably binds to unfolded proteins, was added to the reaction. In
the presence of GroEL-trap, a complex between GroEL-trap and
unfolded GFP polypeptides is formed, therefore preventing GFP reaggregation.

Previously, our kinetic studies suggested that the incubation of aggre-
gated substrate with the K/J/E chaperones is the rate-limiting step of the
disaggregation reaction. Consequently, the delay in formation of prop-
erly folded substrate molecules during the ClpB-K/J/E-dependent dis-
aggregation reaction was postulated to be dependent on the initial
action of K/J/E chaperones on aggregated substrate (31). Nevertheless,
the small aggregates formed upon K/J/E-concerted action are not inter-
mediates in the ClpB-K/J/E-mediated disaggregation reaction. These
aggregates reisolated by a second round of sedimentation still require
both ClpB and the K/J/E chaperone machine for efficient disaggregation
and refolding (results not shown). Moreover, similarly to the large
aggregates, preincubation with the K/J/E chaperone machine is
required to obtain a rapid initiation of disaggregation reaction. There-
fore the small aggregates are most likely dead end products formed only
in the experimental in vitro system lacking ClpB and are not true dis-
aggregation intermediates.

Further, the ability of the K/J/E chaperone machine to disentangle
GFP polypeptides from aggregates does not necessarily mean that dur-
ing disaggregation monomers are actually released into solution. It is
likely that the unfolded substrate still associated with Hsp70 is trans-
ferred to the Hsp100 chaperone before it reaches the native confor-
maion (Fig. 7A). The experiments in which unsuccessful attempts were
made to replace the Hsp100 chaperone by its homologue either from a
different organism or from different cellular compartment (20, 23, 43)
suggest that Hsp70 and Hsp100 chaperones do functionally interact. On
the other hand several attempts to show a direct physical interaction
between Hsp70 and Hsp100 have failed. However, recently it has been
demonstrated that DnaK and ClpB homologues from Thermus ther-
mophilus form a complex (43).

The data presented here place the action of K/J/E leading to the
dissociation of aggregates upstream of ClpB and suggest that ClpB does
not play a direct role in the initiation of disaggregation process. Our
observations are consistent with the recent work from the laboratory of
Bukau and co-workers (32) showing the threading of an unfolded
polypeptide through the ClpB channel as a mechanism of ClpB action.
The presence of such a channel, able to accommodate the unfolded
polypeptide chain, was shown to exist in the structure of ClpB hexamer
from T. thermophilus (33). It was also shown that structural integrity the
channel is required for Hsp104 and ClpB functions (34, 35), supporting
the proposal that ClpB and Hsp104 translocate substrates through the
channel. The threading would result in the unfolding of misfolded
translocated polypeptides. In this way ClpB could prevent the reaggre-
gation of misfolded polypeptides liberated from aggregates by K/J/E
action. By analogy to co-translation folding of a polypeptide chain
emerging from a ribosome, the threading mechanism would allow also
the sequential folding of the polypeptide chain emerging from the cen-
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**FIGURE 7. A model of Hsp70-Hsp100-dependent disaggregation of aggregated proteins.** A, substrate monomerization and refolding in the presence of Hsp70 and Hsp100. Light gray aggregate was drawn to emphasize the possibility that the processing of polypeptide by ClpB chaperone may take place either at the surface of aggregates or in the solution as discussed. B, substrate monomerization and its reaggregation in the presence of Hsp70 only. See “Discussion” for details.

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