Active Solubilization and Refolding of Stable Protein Aggregates By Cooperative Unfolding Action of Individual Hsp70 Chaperones

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Hsp70 is a central molecular chaperone that passively prevents protein aggregation and uses the energy of ATP hydrolysis to solubilize, translocate, and mediate the proper refolding of proteins in the cell. Yet, the molecular mechanism by which the active Hsp70 chaperone functions are achieved remains unclear. Here, we show that the bacterial Hsp70 (DnaK) can actively unfold misfolded structures in aggregated polypeptides, leading to gradual disaggregation. We found that the specific unfolding and disaggregation activities of individual DnaK molecules were optimal for large aggregates but dramatically decreased for small aggregates. The active unfolding of the smallest aggregates, leading to proper global refolding, required the cooperative action of several DnaK molecules per misfolded polypeptide. This finding suggests that the unique ATP-fueled locking/unlocking mechanism of the Hsp70 chaperones can recruit random chaperone motions to locally unfold misfolded structures and gradually disentangle stable aggregates into refoldable proteins.

Hsp70 is the most ubiquitous and abundant molecular chaperone in all organisms with the exception of some Archaea. Most organisms express constitutive and stress-induced forms of Hsp70s, which are involved in protein folding, translocation, and unfolding in the various ATP-containing compartments of the cell. Hsp70 serves as a central element of the chaperone network, assisting in de novo protein folding, protein translocation, and oligomer dissociation as well as in the passive prevention of protein aggregation and the active recovery of stress-induced protein aggregates (1–3).

Hsp70 is composed of two major domains: an actin-like N-terminal ATPase domain and a C-terminal domain containing a substrate-binding cleft covered by a flexible lid. The substrate-binding domain of bacterial Hsp70 (DnaK) can typically bind short hydrophobic motives flanked by positive charges in extended peptides in partially unfolded or misfolded proteins (4–6). Hsp70 alternates between an ADP-bound substrate-bound locked state and an ATP-bound substrate-bound unlocked state (7). ATP hydrolysis, which is catalyzed by co-chaperone Hsp40 (DnaJ in bacteria), induces the tight closure (locking) of the lid and the transient increase of the chaperone locked state (7). ATP hydrolysis, which is catalyzed by co-chaperone GrpE in bacteria, induces the opening (unlocking) of the lid and dissociation of the Hsp70 from the substrate (7, 9).

The molecular mechanism by which a highly conserved molecule such as Hsp70 may perform a wide array of apparently dissimilar cellular functions remains unclear. Despite progress in understanding the Hsp70 cycle (7), it is not known how ATP-fueled alternate cycles of Hsp70 binding/release to and from misfolded or partially unfolded proteins can actively translocate polypeptides into organelles, drive the dissociation of native oligomers, and solubilize stable protein aggregates into native proteins (10). Here, we used sedimentation, fluorescence spectroscopy, and kinetic analysis to address the molecular mechanism by which the E. coli Hsp70 (DnaK) and its co-chaperones, DnaJ and GrpE, can mediate the active solubilization and refolding of stable heat-generated protein aggregates. Our data fit a mechanism whereby local unfolding of exposed protein segments in misfolded aggregates is achieved by independent random motions of several individual DnaK molecules tightly bound to the same misfolded polypeptide. Upon nucleotide and chaperone release, unfolded protein segments may properly refold, preventing chaperone rebinding, or improperly refold, allowing chaperone rebinding. Such iterative cycles of local unfolding by bound chaperones and spontaneous local refolding upon chaperone release may gradually solubilize and lead to the stepwise refolding of stable misfolded aggregates into native proteins.

EXPERIMENTAL PROCEDURES

Heat Inactivation and Aggregation—Glucose-6-phosphate dehydrogenase (G6PDH) from Leuconostoc mesenteroides (Sigma) was incubated for 15 min at 52 °C in buffer A (100 mM Tris, pH 7.5, 150 mM KCl, 20 mM MgAc2, and 10 mM dithiothreitol). The residual activity after the inactivation was <10%, and no spontaneous reactivation was observed.

Chaperone Reactivation Assays—Chaperone-mediated reactivation assays were carried at 25 °C in the presence of chaperones as indicated in buffer A supplemented by 4 mM ATP and an ATP regeneration system (5 mM phosphoenolpyruvate and 20 mM/mg pyruvate kinase). The enzymatic activity of G6PDH was measured at 25 °C as described previously (11). All of the protein concentrations are expressed in protomers, regardless of the oligomeric state of the chaperones or the aggregates. Chaperone/substrate ratios are protomer ratios.

Unfolding Measurements—The time-dependent unfolding of pre-aggregated G6PDH in the presence of chaperones as in Fig. 1a and of Thiolavfin-T (10 μM) was monitored continuously for 1 h at 25 °C in a PerkinElmer Fluorometer (LS50B) (excitation at 400 nm and detection at 480 nm).

Gel Filtration Chromatography—The apparent sizes of the denatured G6PDH were estimated by size-exclusion chromatography in low salt buffer (5 mM Tris, pH 7.5) using a Superose-6B gel filtration column.

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The abbreviation used is: G6PDH, glucose-6-phosphate dehydrogenase.
(Amersham Biosciences) at a flow rate of 0.5 ml/min. Samples were concentrated, and proteins were resolved by SDS-gel electrophoresis. The amounts of soluble proteins were estimated from the intensity of Coomassie Blue-stained bands using the NIH gel-scanning program. Apparent molecular masses were estimated using gel-filtration standards from Bio-Rad and the native 120-kDa G6PDH dimer.

**Solubility Fractionation by Sedimentation—**Solubility fractionation of protein aggregates was evaluated from the quantity of proteins present in the soluble fraction after a 10-min centrifugation at the indicated velocities (4,000–228,000 ×g) using a Beckman ultracentrifuge with a TLA-100.2 fixed angle rotor. Soluble proteins were resolved by SDS-gel electrophoresis, and the amounts of soluble proteins were determined as above. The results in Fig. 2, a and b, are expressed as the fraction (%) of soluble protein after centrifugation compared with the total protein amount before centrifugation as described previously (11).

**RESULTS**

**Cooperation between DnaK Molecules Is Required for Refolding of Aggregates—**When the model enzyme G6PDH is heat-denatured (52 °C, 15 min) in the absence of chaperones, it forms stable inactive aggregates, mostly soluble at low-speed centrifugation (11). As initially shown by Diamant et al. (10), such aggregates are very stable. They do not spontaneously refold nor do they dissociate, and their oligomeric state is insensitive to extensive dilutions and gel filtration. The addition of a molar excess of DnaK over the aggregate of co-chaperones (DnaJ and GrpE in equimolar amounts as the substrate) and ATP resulted in a significant reactivation of the G6PDH enzyme (10). Remarkably, a 10-fold molar excess of DnaK over the substrate (protomer to protomer) efficiently reactivated half of the G6PDH within 20 min, whereas equimolar DnaK (with co-chaperones) remained refolding-inefficient (Fig. 1a). A DnaK dose-response (with constant DnaJ and GrpE) confirmed that DnaK-mediated refolding required the chaperone to be in molar excess over its substrate (Fig. 1b). In a 10-fold molar excess of chaperone, the specific refolding activity of DnaK (expressed in rate per DnaK) was four times higher than when at an equimolar amount with the substrate (Fig. 1b). This finding suggests that a concerted action among several DnaK molecules on the same misfolded polypeptide is required for effective refolding. When the same DnaK dose response was performed in the presence of the disaggregating co-chaperone ClpB (6), the reactivation rates were expectedly higher (up to 6-fold) than without ClpB (Fig. 1b, inset) and the apparent affinity of DnaK for the aggregate was increased by a factor of 5 (Fig. 1b). Yet, equimolar DnaK (with respect to the substrate protomers) remained as ineffective with ClpB as without ClpB (Fig. 1b), indicating that, regardless of the synergism in the disaggregating mechanism of ClpB, the requirement of a molar excess of DnaK over the substrate for effective refolding is an intrinsic property of the Hsp70 mechanism.

We next addressed the possibility that the cooperative behavior of DnaK in active aggregate refolding is carried by a discrete DnaK oligomer in which the DnaK monomers are directly associated and influence each other’s activity. Hence, to preserve the stability of a presumed chaperone oligomer, we kept the chaperone concentration high and constant and the specific refolding activity was measured in the presence of decreasing concentrations of the substrate (Fig. 1b). Whereas in the absence of chaperones or ATP dilution had no effect on the stability and reactivity of the aggregates (10, 11), the presentation of dilute aggregates to a constant high concentration of DnaK (and of DnaJ, GrpE, ClpB, and ATP) resulted in a net increase of the chaperone-specific refolding activity. At equimolar (protomer to protomer) amounts of chaperone/substrate, the specific refolding activity was very low but it became optimal when the molar ratio was restored by aggregate dilution to a 5-fold chaperone excess (Fig. 1b). This behavior is contrary to a classic enzymatic reaction, in which substrate dilution may only maintain or reduce the specific activity of a fixed concentration of enzyme. The observed increase in the chaperone-specific activity could not be attributed to dilution-induced changes in the substrate, as heat-denatured G6PDH aggregates are not dynamic (10, 11). Rather, the specific refolding activity of the chaperone appeared to depend on its ratio with the substrate. Regardless of the absolute concentrations, a constant molar excess of chaperones over the substrate was essential for effective native refolding. These observations speak against an active DnaK oligomer in which DnaK subunits are in direct contact and functionally cooperate with one another as in the case of the dilution-sensitive ClpB6 and GroEL14 oligomers. At the same time, the dependence of the specific refolding activity on a molar excess of chaperone over the substrate clearly confirms that the cooperative action of several Hsp70 molecules is necessary for the active conversion of stable misfolded complexes into correctly refolded proteins. Because proteins contain hydrophobic segments on average every 30–40 residues to which DnaK can specifically bind (4), it is possible that several individual DnaK monomers can independently bind at different sites on the same misfolded polypeptide. Our results indicate that, by sharing a common polypeptide substrate, several DnaK molecules may cooperate in the active unfolding of the substrate without having necessarily to form a discrete chaperone oligomer.

**Disaggregation Activity depends on the size of the aggregates—**We next addressed the kinetics of two DnaK-dependent activities that precede chaperone-mediated protein refolding:
active solubilization and unfolding of the stable protein aggregates. Sedimentation analysis at different velocities allowed us to follow in time the changes in the solubility profile of the aggregates at different size ranges following treatment with different DnaK concentrations. As expected for stable aggregates that are not in dynamic equilibrium, the distribution of aggregate sizes remained unchanged within 24 h following extensive dilutions or in the absence of chaperones (10, 11). Yet, a 150-min incubation with equimolar DnaK (ATP and constant co-chaperones) resulted in a significant solubilization (30–40%) of the aggregates in all of the size ranges (Fig. 2a) albeit without the recovery of active G6PDH enzymes. Gel-exclusion chromatography confirmed that partial solubilization of the aggregates took place with a significant increase of smaller inactive species, particularly species eluting at the same position as the native dimer and at a position expected for inactive monomer (double and single arrows, respectively, Fig. 2a, inset). Therefore, in the presence of equimolar amounts of DnaK (ATP and co-chaperones), large aggregates were actively fragmented and accumulated as smaller species, possibly misfolded dimers and monomers, which were not converted further into native dimers (Fig. 1a).

In an attempt to visualize in time the DnaK-mediated disaggregation, we used fractionation by sedimentation at various velocities (Fig. 2b). Under treatment with a constant equimolar concentration of DnaK (and of co-chaperones and ATP), a fraction corresponding to very large aggregates (sedimenting at the lowest speed, 14,000 × g) readily became soluble. In contrast, another fraction corresponding to medium size aggregates (sedimenting at 128,000 × g) became soluble only after 40 min. The smallest aggregates, sedimenting at 228,000 × g, became more soluble only following 2 h of active chaperone treatment (Fig. 2b). This corroborates our observation that, with equimolar DnaK, small aggregates form and accumulate but do not refold (Figs. 1a and 2a) and demonstrates that the specific disaggregation activity of DnaK decreases with the size of the aggregates.

A DnaK dose-response analysis of the rates of aggregate solubilization confirmed that different degrees of cooperativity between the DnaK molecules were needed for the effective solubilization of different oligomeric states of the substrate (Fig. 2c, inset). Whereas sub-stoichiometric amounts of chaperones could optimally fragment the largest aggregates into smaller ones, the solubilization of smaller aggregates required increasing molar excess amounts of the chaperone over the substrate (Fig. 2c). This strongly indicates that the same chaperone mechanism is responsible for disaggregation and refolding, although increasingly cooperativity between Hsp70 molecules is needed to complete the reaction as the oligomeric state of the aggregates decrease. The highest molar excess of chaperones (EC50 ~5) was needed to carry out the final stage of the reaction corresponding to the active conversion of stable misfolded monomers into natively refoldable ones.

The ability of the chaperone to discriminate between small and large aggregates explains the accumulation of small inactive species observed when DnaK was at limiting concentrations. Although, during the initial phases of the reaction, the largest aggregates were first efficiently fragmented, in the later phases of the reaction, smaller aggregates were or were not further converted into smaller ones because of the size-dependent decrease in the chaperone efficiency. Increasing chaperone amounts could compensate for the size-dependent decrease in DnaK efficiency and allow the reaction to reach its final outcome of correct proper refolding.

**DnaK Actively Unfolds Misfolded Structures in Aggregates—** The fact that, by an ATP-dependent mechanism, DnaK can actively convert stable aggregated structures into alternatively stable native structures already suggests that the chaperone is mainly involved in unfolding misfolded proteins (1). Here, we directly demonstrated the unfolding activity for DnaK using fluorescence spectroscopy in the presence of Thioflavin-T, a dye that specifically binds β-sheet structures, which are enriched in misfolded and aggregated proteins (12). Under the tested conditions, heat-aggregated G6PDH (1 μM) (as in Fig. 1a) bound ~75% more Thioflavin-T (10 μM) than native G6PDH,
confirming that the aggregated form contains a higher amount of amphiphilic intramolecular and intermolecular β-sheet structures, as in the case of amyloid aggregates and prion particles (12). In the presence of a 9.4 molar excess of DnaK (with constant DnaJ and GrpE and ATP), a rapid time-dependent decrease of Thioflavin-T binding was observed in the aggregates (Fig. 3a) but not in the native controls or when the chaperone, co-chaperone, or ATP was omitted (data not shown). Moreover, indicating that DnaK can unfold a wide array of misfolded substrates, we found that an excess of DnaK (with co-chaperones and ATP) strongly decreased the signal of bound Thioflavin-T and that at the same time it increased the solubility of heat-aggregated proteins from a total E. coli extract (data not shown).

A DnaK dose response (with constant ATP, DnaJ, and GrpE) of the optimal unfolding rates of G6PDH (Fig. 3b, inset) revealed that the half-maximal unfolding velocity was reached in the presence of a 5-fold (molar) excess of the chaperone over the substrate. This confirms that DnaK-mediated unfolding activity also requires the cooperative action of several DnaK molecules but less than that for DnaK-mediated refolding activity (Fig. 1b). Moreover, at an equimolar DnaK/substrate, the unfolding activity was four times higher than in the presence of a 10-fold excess of DnaK (Fig. 3b), which is different than in the case of the refolding activity (Fig. 1). This strongly indicates that the unfolding activity, leading to the rapid disaggregation of large aggregates and to the less efficient disaggregation of smaller ones, is optimal when carried out by individual DnaK molecules. In contrast, the refolding activity, resulting from the optimal unfolding of misfolded monomers, appears to require the highest degree of cooperation between several individual DnaK molecules bound to the same misfolded substrate. The fact that, at equimolar chaperone concentration, unfolding was highly efficient but did not result in productive refolding of the substrate implies that the final unfolding events leading to refolding are limited by the reduced size of the substrate.

The Role of Co-chaperones in Unfolding and Disaggregation—We next addressed the role of DnaJ, which accelerates ATP hydrolysis in DnaK and the locking of DnaK onto its substrate, and that of GrpE, which accelerates ADP release from DnaK and the release of DnaK from its substrate (7, 8), in the productive refolding of stable protein aggregates. A DnaJ dose response on the rates of DnaK-mediated refolding of stable aggregates (as in Fig. 1) was performed without and with GrpE (Fig. 4). Surprisingly, our stringent in vitro assay of DnaK-DnaJ-mediated refolding of heat-preaggregated G6PDH was found to be active, even in the absence of GrpE albeit at slightly lower rates but not in the absence of DnaJ (Fig. 4). Therefore, ADP release from DnaK and the unlocking of DnaK from its substrate can occur spontaneously, although by accelerating ADP release, GrpE seems to optimize DnaK as an active unfolding machine. In contrast, transient DnaJ-mediated locking with very high affinity of DnaK onto its substrate is central to the unfolding, disaggregation, and refolding activities of the chaperone.

Optimal refolding rates were observed at a DnaJ:DnaK ratio of 0.04, yet half of the optimal refolding rates were obtained at an EC_{50} of 0.014 (Fig. 4), implying that for optimal chaperone unfolding/refolding activities, a single DnaJ molecule can act as a locking catalyst for as many as 70 DnaK molecules. When DnaJ was at an optimal physiological ratio with DnaK of 0.2, the EC_{50} value for GrpE was 0.057 (Fig. 4, inset), indicating that, during the chaperone cycle, a single GrpE dimer can act as an unlocking catalyst for dozens of DnaK molecules. Thus, both DnaJ and GrpE behave as catalysts of the chaperone functional cycle by, respectively, regulating the phase during which DnaK is tightly bound and locally unfolds the substrate and the phase during which DnaK is unbound and local correct refolding can occur spontaneously.

**DISCUSSION**

**Passive and Active Molecular Mechanisms of the Hsp70 Chaperones**—The classic activity attributed to molecular chaperones is the ability to bind and prevent early misfolding intermediates from irreversible aggregation and thus, upon chaper-
erone dissociation, promote the proper refolding of the substrate proteins (14). Indeed, Hsp70 (and possibly also Hsp40) may passively bind unstable misfolding intermediates, thereby reducing the extent of protein aggregation and improving subsequent refolding reactions (1). However, stable preformed protein aggregates can also be directly scavenged and correctly refolded by Hsp70, co-chaperones, and ATP, implying that prevention of aggregation is not an absolute requisite of the Hsp70 chaperone activity (1, 6, 10). Similarly, when imported into mitochondria, cytosolic polypeptides are unfolded by an active, ATP-consuming, Hsp70-dependent mechanism that does not seem to involve passive prevention of aggregation but unfolding by the Brownian motions of Hsp70 (15). Thus, although passive prevention of aggregation is an important function associated with many molecular chaperones, it is optional and cannot account for all of the ATP-dependent functions of Hsp70 chaperones in the cell.

We found here that the unfolding of misfolded structures is a new ATP-dependent catalytic activity that is to be ascribed to Hsp70 chaperones. Alternating cycles of Hsp70 binding, active unfolding, and chaperone release can mediate the ATP-consuming conversion of a stable misfolded aggregated state into a transient unstable state (a partially unfolded state), which then may spontaneously acquire an alternatively stable state (the native state).

Unfolding, disaggregation, and reactivation exhibited different optima in terms of Hsp70-specific activity. This differential behavior indicated that, while only a minimal amount of optimally efficient unfolding events may readily lead to the fragmentation of large aggregates into medium-sized aggregates, the subsequent fragmentation of medium-sized aggregates into the smaller ones are gradually less efficient. Finally, we found that efficient unfolding of the smallest aggregate species into a form that can natively refold, requiring a concerted unfolding action carried by several chaperone molecules per substrate.

DnaK is known to preferentially bind unstructured peptide segments with a hydrophobic core of 4–5 residues flanked by basic residues (4), which are normally buried in native proteins. Because in aggregates some hydrophobic segments participate in oligomeric interactions, the relative amount of the exposed available DnaK-binding sites is expected to decrease proportionally to the size of the aggregate. Yet, here we found that the specific disaggregation activity of Hsp70 increased with the size of the aggregate, suggesting that the size of the aggregates plays a predominant role in the mechanism of Hsp70-mediated unfolding despite better sequestration of potential Hsp70-binding sites in larger aggregates.

Cooperative Unfolding Can Be Achieved by Individual Hsp70 Molecules—The chaperonin oligomers act as power-stroke machines in which several GroEL subunits cooperate at binding and disrupting misfolded structures within a bound substrate polypeptide, leading upon release to the proper refolding of the latter (16). We addressed the possibility that a similar mechanism is carried by a cooperative Hsp70 oligomer. However, incompatible with a mechanism involving a discrete oligomer, we found that the specific unfolding, disaggregation, and refolding activities of DnaK depended on different degrees of cooperativity among chaperone molecules, on the size of the substrate, and on the chaperone/substrate ratio. In a discrete active oligomer, the specific disaggregation activity should only depend on the affinity to the substrate and on the absolute chaperone concentration but not on the chaperone/substrate ratio nor on the size of the aggregate as in our findings.

Rather, cooperative unfolding of aggregates may be achieved by a different type of cooperative Hsp70 complex in which individually bound Hsp70 molecules indirectly cooperate with each other by a common misfolded-polypeptide substrate that they may share. Such a situation has been already suggested in the case of protein import into the mitochondria in which the cooperative ratchet action of several individual Hsp70 molecules that bind to the same imported polypeptides may produce a net unidirectional protein transport (15).

Our observation is in agreement with most in vitro studies where Hsp70 was monomeric in the presence of ATP (17, 18). Moreover, there is no structural evidence that Hsp70 molecules can form discrete functional oligomers with or without Hsp40 that would be able to bind ends of a misfolded polypeptide and apply unfolding force upon them by a power-stroke. Although in Thermus thermophilus some DnaK is found associated in a trigonal complex with DnaJ and the assembly factor TdaFA, the active form of the chaperone is the free DnaK monomer when the trigonal complexes are dissociated at physiological 55 °C (18).

The Role of Co-chaperones Supports Unfolding by Chaperone Random Motions—We found that both DnaJ and GrpE act as catalysts of the chaperone functional cycle by regulating the time DnaK spends in the tightly bound state during which productive unfolding by random motions may take place and by regulating the time DnaK is in the unbound state during which productive spontaneous local refolding may take place (Fig. 4). This is further demonstrated at higher concentrations of DnaJ (0.175:1 DnaJ:DnaK) where there is an inhibition of the chaperone dissociation, protein import into the mitochondria in which the cooperative ratchet action of several individual Hsp70 molecules that bind to the same imported polypeptides may produce a net unidirectional protein transport (15).

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be alleviated by GrpE and confirming its activity as a chaperone release factor. The role of DnaJ and GrpE, respectively, catalyzing the tight locking of DnaK and its subsequent release to and from the misfolded substrate is fully compatible with an unfolding mechanism based on the random motions of individual DnaK molecules bound to a common misfolded substrate. By mediating the tight locking of DnaK onto the substrate, DnaJ can harness the energy from ATP hydrolysis to an efficient pulling force to be exerted by the random movements of tightly locked DnaK molecules. DnaK unlocking and dissociation, allowing the spontaneous local refolding of the substrate, are greatly optimized by GrpE, especially in the presence of high physiological concentrations of DnaJ, which would otherwise retard chaperone release (Fig. 5a).

Coupling ATP Hydrolysis to Unfolding—In order for locally bound individual DnaK molecules to successfully unwind misfolded protein structures by random motions, the binding energy of the chaperone must exceed that of average misfolded structures or else movements would lead to an unproductive dissociation of the chaperone. DnaK or DnaK-ATP can bind with a low but not insignificant affinity to short hydrophobic peptides. ATP hydrolysis strongly increases the binding energy (4, 5, 7), and consequently, peptide release from DnaK-ADP may be more than a thousand times slower than from DnaK-ATP (19). Because the binding energy is proportional to the logarithm of the binding time, the affinity of DnaK-ADP for the polypeptide must be larger than that of DnaK-ATP by roughly 7 kT. Highly stable native domains may require up to 20 kT for their unfolding, but randomly misfolded domains are expected to be less specifically structured and therefore less stable than native domains. Therefore, the high energy in the transient bond between DnaK-ADP and a misfolded domain may well withstand a random pulling force in the same order as the force necessary to unfold it.

The Cooperative Unfolding Cycle of Hsp70—The ability of Hsp70 to cycle between the two states by ATP hydrolysis (unlocked-to-locked) and ADP release (locked-to-unlocked) is key for its function as a cooperative protein-unfolding machine. In agreement with previous studies (7), our data suggest that the active chaperone cycle of Hsp70 involves the following steps (Fig. 5a): 1) weak binding of Hsp70-ATP to an exposed hydrophobic motif in a misfolded polypeptide; 2) DnaJ-activated ATP hydrolysis, causing tight locking of Hsp70 onto the misfolded polypeptide; 3) unfolding of the bound segment by random movements of Hsp70; 4) GrpE-activated ADP release, causing the unlocking of Hsp70 from the unfolded segment; and 5) spontaneous refolding of the unfolded segment into a more native structure for which Hsp70 has a lower affinity. Hsp70 may then rebind to another misfolded and exposed hydrophobic motive in the same polypeptide, thus gradually unfolding and correctly refolding various misfolded regions in the aggregate. Initial unfolding can lead to polypeptide disentanglement from the aggregate. In the last step of the unfolding reaction, several Hsp70 molecules are needed to bind to the same misfolded polypeptide because only the cumulative, independent motions of several bound Hsp70 molecules randomly moving in opposite directions can sum up in the effective unfolding of single polypeptide into a form that can spontaneously refold into a native protein (Fig. 5b). Alternatively, it is possible that the last steps of the unfolding reaction may still be carried out by a majority of Hsp70 molecules when the terminal-misfolded species are maintained in a complex with other power-stroke chaperones such as ClpB (20) and GroEL (21) or holding chaperones such as small heat shock proteins (22).

Implications for Pathological Aggregates—In the cytoplasm of mammalian cells, there are no ClpB and GroEL homologues that can increase the efficiency of Hsp70 and the entire burden of aggregate detoxification must relay on Hsp70. Because Hsp70 molecules are needed to complete solubilization and refolding (Fig. 1) (1), it is essential that mammalian cells anticipate the synthesis of an excess amount of the chaperone prior to the formation of pathological aggregates. This is normally achieved by competition between heat shock factors and the Hsp70 substrates (22). However, when heat shock factor-induced chaperone synthesis is impaired as in aging or when misfolded species form too rapidly as a result of stress or mutations, pathological accumulations of misfolded species may occur.

The pathology of misfolding diseases indicates that the smallest misfolded species are more toxic (24, 25). Our results describe a situation in which insufficient amounts of Hsp70 in the cell may convert large inert aggregates into smaller, potentially more toxic and infectious particles without being able to further convert them into harmless native proteins. Therefore, the relative concentration of Hsp70 must exceed that of pathological aggregates for effective curing. Indeed, the overexpression of Hsp70 in several model systems has resulted in the clearance of pathological aggregates and the arrest of the symptoms (26). Therefore, understanding the mechanism of Hsp70 is crucial in the design of a strategy for safe and effective treatments of protein-misfolding diseases.

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