Proteinaceous Infectious Behavior in Non-pathogenic Proteins Is Controlled by Molecular Chaperones

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External stresses or mutations may cause labile proteins to lose their distinct native conformations and seek alternatively stable aggregated forms. Molecular chaperones that specifically act on protein aggregates were used here as a tool to address the biochemical nature of stable homo- and hetero-aggregates from non-pathogenic proteins formed by heat-stress. Confirmed by sedimentation and activity measurements, chaperones demonstrated that a single polypeptide chain can form different species of aggregates, depending on the denaturing conditions. Indicative of a cascade reaction, sub-stoichiometric amounts of one fast-aggregating protein strongly accelerated the conversion of another soluble, slow-aggregating protein into insoluble, chaperone-resistant aggregates. Chaperones strongly inhibited slow-induced protein aggregation, suggesting that they can prevent and cure proteinaceous infectious behavior in homo- and hetero-aggregates from common and disease-associated proteins in the cell.

Protein structure is a compromise between the dictates of structural stability and functional flexibility. Principally driven by hydrophobic interactions, protein aggregations arise from the association of partially structured unfolding intermediates that seek alternatively stable but poorly defined conformations (1, 2). In mutant proteins, or when the temperature is raised above the physiological optimum, some domains in labile proteins may lose their structure and seek alternatively stable structures, whereas other more compact domains may still retain their original native-like conformation (1, 3). In general, stable aggregates lack enzymatic activity, expose more hydrophobic residues, have a higher $\beta$-sheet content, and adopt high oligomeric, less soluble quaternary structures (1, 4). Some aggregates display an amorphous organization, whereas others, such as amyloid plaques, form compact fibrils reaching highly ordered semi-crystalline structures (5, 6). Aggregation-prone proteins tend to form insoluble fibrils implicated in protein conformational disorders such as polyglutamine diseases, amyloidosis, and prion diseases (5–8).

Protein aggregates that accumulate under heat-stress in vitro, in bacteria, or in yeast can be selectively resolubilized by a specific network of ATP-hydrolyzing chaperones and co-chaperones: Hsp100 (ClpB) and Hsp70/Hsp40/Bag1 (DnaK, DnaJ, GrpE) (Escherichia coli chaperones in parentheses) (9–11). The disaggregation mechanism involves a sequential action, first of ClpB modifying the surface of large compact aggregates thus increasing DnaK affinity, and second, of DnaK binding and unfolding entangled polypeptides, which accelerates cis-trans isomerizations in secondary amide bonds of refolding polypeptides (12, 13). The efficiency of chaperone-mediated disaggregation strongly depends on the nature of the protein, on the aggregate size, and its solubility (4, 10, 11).

Disaggregating chaperones were used here as a tool to address the nature of homo- or heterogeneous protein aggregates, using three model proteins: glucose-6-phosphate dehydrogenase (G6PDH),1 malate dehydrogenase (MDH), and bovine serum albumin (BSA). Confirmed by sedimentation, chaperones revealed that non-pathogenic proteins can produce different forms of heat-induced aggregates. Aggregates from one protein can associate with and accelerate the aggregation of another protein, a proteinaceous infectious behavior, which can be prevented and cured by molecular chaperones.

EXPERIMENTAL PROCEDURES

Proteins—Protein purification was according to published procedures: DnaK, DnaJ, GrpE, (14); ClpB (15); HspB (16). Malate dehydrogenase from pig heart mitochondria was from Roche Molecular Biochemicals. Rabbit muscle pyruvate kinase, Leuconostoc mesenteroides glucose-6-phosphate dehydrogenase, and bovine serum albumin were from Sigma. Protein concentrations were determined using the Bio-Rad Bradford assay with BSA as a standard. Protein concentrations were expressed in protomers.

Protein Aggregation and Precipitation—Aggregates were formed by heat inactivation of the native proteins for 15 min, unless specified otherwise, and at the indicated temperature (52–75 °C) in folding buffer (100 mM Tris-HCl, pH7.5, 150 mM KCl, 20 mM MgAc, 10 mM dithiothreitol). Unless indicated otherwise, aggregation was preformed with 1 μM protein. Stable aggregates of MDH, G6PDH, and BSA were combined before, during, or after the heat treatment, as indicated.

Fractionation of insoluble/soluble aggregates was preformed either by a 20-min centrifugation at 15,800 × g in an Eppendorf tabletop centrifuge (5417R), or by a 10-min centrifugation at various velocities (up to 365,000 × g) in a Beckman ultracentrifuge using a TLA-100.2 fixed angle rotor. Precipitated MDH, G6PDH, or BSA was resolubilized in 5 M urea and SDS-sample buffer containing 2% $\beta$-mercaptoethanol and 10% SDS. Following 3 min of incubation at 80 °C, samples were loaded and resolved by electrophoresis on a linear 15% SDS-polyacrylamide gel. The amount of precipitated and soluble protein was measured from the intensity of Coomassie Blue-stained bands using the NIH gel-scanning program. Amounts were normalized to maximal precipitation values obtained at 75 °C (15 min) or to the amount of native protein.

Chaperone Activity Assays—Chaperone-mediated reactivation was carried out in folding buffer at 25 °C in the presence of an ATP regeneration system (4 mM phosphoenolpyruvate, 20 mM pyruvate kinase).

Unless specified otherwise, chaperone concentrations were 2 μM DnaK.

1 The abbreviations used are: G6PDH, glucose-6-phosphate dehydrogenase; KJE, DnaK + DnaJ + GrpE; MDH, malate dehydrogenase; BSA, bovine serum albumin; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid.
One Protein Can Generate Different Aggregates—A central question about protein aggregation is whether single polypeptide chains form one or several types of protein aggregates during and following stress-induced denaturation. To address this question, different aggregates from a single enzyme, G6PDH, were generated by a 15-min exposure to various high temperatures (52–75 °C). When denatured at 62 °C, G6PDH was rapidly inactivated at a rate of 0.045 s⁻¹ (Fig. 1A). After 15 min, large insoluble aggregates were formed (Fig. 1B), half of which (EC₅₀) required 3,000 × g to be precipitated (Fig. 1C). When denatured at 52 °C, G6PDH was inactivated 10 times more slowly, and it formed inactive more soluble aggregates, half of which required as much as 175,000 × g to be precipitated (Fig. 1C).

Using disaggregating chaperones, we then compared the refoldability at 25 °C of each type of aggregate. In the presence of a limiting amount of chaperones and ATP, the more soluble aggregates generated at 52 °C were reactivated four times faster than the less soluble aggregates generated at 62 °C (Fig. 1D). The later were, however, fully reactivated following longer incubations or in the presence of excess chaperones (see below), demonstrating the lack of irreversible lesions in the 62 °C-treated protein.

Differences between various types of G6PDH aggregates were also observed using a hydrophobic fluorescent probe, Bis-ANS. The higher the temperature at which aggregates were pre-formed (and the lower was their solubility), the higher was their resistance to disaggregating chaperones after the stress (Fig. 1E, inset). Noticeably, chaperone-resistance increased with the apparent hydrophobic exposure of the aggregates (Fig. 1E, inset). Because DnaK binding primarily depends on exposed hydrophobic segments in misfolded proteins (18), this suggests that disaggregation activity of ClpB in particular depends on other properties of the aggregates.

Heterologous Protein Aggregations—The presence of misfolded proteins has been suggested to initiate and sustain aggregation of other stress-induced unfolding intermediates (19). We therefore next addressed protein aggregation in a more physiological context, where several proteins co-exist and may co-aggregate. MDH is more thermostable than G6PDH. Within 6 min at 52 °C, over 90% became insoluble at 15,800 × g (Fig. 2A) compared with 15% insoluble G6PDH (Fig. 1A). The effect of a rapidly aggregating MDH species on the aggregation kinetics of an otherwise slow-aggregating soluble G6PDH species (Fig. 2A) was addressed at 52 °C. The presence of equimolar amounts of MDH did not affect the rate of G6PDH inactivation but, remarkably, strongly decreased the solubility of G6PDH aggregates (Fig. 2B). The presence of five times less MDH molecules than G6PDH reduced the G6PDH precipitation values (EC₅₀) from 171,000 × g to 15,000 × g (Fig. 2C). This effect was not observed when MDH was pre-aggregated and added to
G6PDH after the stress (Fig. 3A). At various centrifugation velocities, the distribution of G6PDH aggregates co-aggregated with MDH revealed a sharp decrease of solubility, by half for the largest aggregates and up to 80% for the smallest aggregates (Fig. 2C). Thus, all sizes of G6PDH aggregates were affected by the presence of MDH.

A reciprocal mild solubilization effect of the MDH aggregate by co-aggregating G6PDH was observed. Whereas only 100 g sufficed to precipitate half the MDH aggregates alone, 4,000 g were necessary to precipitate the same amount of co-aggregated MDH in the presence of a 5-fold excess of more soluble
G6PDH aggregates (Fig. 2C). This apparent solubilization effect independently demonstrates that stable MDH-G6PDH hetero-oligomers did form during co-aggregation.

Remarkably, alongside with acceleration of aggregation kinetics, chaperone-mediated disaggregation was also impaired in hetero-aggregates. Hence, when MDH and G6PDH in equimolar amounts were co-aggregated, the rates of chaperone-mediated reactivation was eight times slower for MDH-G6PDH hetero-aggregates than for G6PDH homo-aggregates formed at the same temperature (Fig. 3A). In contrast, when in a control experiment, preformed MDH and G6PDH homo-aggregates were mixed after heat treatment, the original solubility and chaperone-recoverability of either protein remained unaffected (Fig. 3A). Hence, compared with homologous aggregation, heterologous aggregation affects both solubility and chaperone recoverability.

**Seeding of Protein Aggregation**—To determine the nature of the interactions between two proteins, leading to decreased solubility and chaperone recoverability, a dose-response of G6PDH precipitation and chaperone recoverability was performed in the presence of increasing concentrations of MDH. Unexpected from one-to-one hetero-aggregates, sub-stoichiometric amounts of MDH fully sufficed to carry both effects. As little as one 30-kDa MDH molecule sufficed to accelerate the precipitation and inhibit chaperone recovery of 4–5 60-kDa G6PDH molecules (Figs. 2C and 3A). In a reciprocal experiment, the presence of sub-stoichiometric amounts of G6PDH seeds (pre-aggregated at 52 °C) affected the MDH solubility and chaperone recoverability during and following a mild denaturation at 43 °C (Fig. 3B). Induced MDH precipitation was confirmed using pre-aggregated seeds from a totally different protein, BSA, which is widely used as a stabilizing agent for labile proteins in vitro. Whereas at 56 °C, native BSA expectedly reduced the rate of MDH inactivation and aggregation (not shown), the presence of BSA in the aggregation rendered MDH aggregates significantly more resistant to disaggregating chaperones after the stress (Fig. 3C). It is concluded that the ability of aggregates from one protein to influence, and even propagate, aggregation of another protein is a general property shared by many proteins, unrelated to amyloidogenic and prion proteins.

**Chaperones Control Seed-induced Aggregation**—Most chaperones can bind partially unfolded intermediates or small aggregates and thus prevent the formation of larger aggregates during stress. Hence, the role of binding chaperones on seed-induced MDH aggregation was addressed. The presence of DnaK (in binding-only mode without ATP) or the small heat shock protein IbpB efficiently inhibited seed-induced precipitation of MDH during mild denaturation at 43 °C (Fig. 4A). As previously shown in Fig. 3B, the MDH solubility with a 0.5 μM G6PDH seed, but without chaperones, was ~30% (~70% precipitation). Yet, the non-catalytic binding effect of equimolar DnaK, or of IbpB chaperones on MDH solubility in the presence a G6PDH seed, was increased to 60 and 47%, respectively (Fig. 4A). A mild increase was also observed in the solubility of the G6PDH seed (not shown). It can be concluded that potential cascade interprecipitation events among different proteins may be prevented by binding chaperones capable of sequestering seeds and aggregation substrates.

During a prolonged stress, the binding capacity of the chaperone network may be overwhelmed (20). The effect of active disaggregation by ClpB and KJE was therefore tested on the outcome of seed-induced aggregation. G6PDH treated at 52 °C in the presence of MDH seeds (as in Fig. 3A), was refolded after the stress by limiting amounts of chaperones at a slower rate than G6PDH treated alone at the same temperature but at a faster rate than G6PDH treated alone at a higher temperature, 62 °C (Fig. 4B). As expected from the higher oligomeric state of seeded aggregates (Fig. 2C), a ClpB dose-response on refolding rates revealed that ClpB has a lower apparent affinity (EC₅₀ 0.15) for seeded G6PDH, than for G6PDH alone (EC₅₀ 0.09) at 52 °C. The recovery of G6PDH prepared at 62 °C depended even more on ClpB (EC₅₀ 0.22), indicating that cross-seeding can mimic the damaging effects of higher temperatures and
generally increase the requirement by aggregating proteins for molecular chaperones.

**DISCUSSION**

**One Protein Can Generate Different Forms of Homo-aggregates**—Depending on cellular and environmental conditions, active flexible proteins may populate a number of very similar native structures (21). Aggregated proteins are often more rigid, less soluble, and tend to assemble into a wide continuum of dissimilar high oligomeric structures. Hence, non-pathogenic proteins that normally form amorphous aggregates can be induced to form highly structured amyloid-like fibrils (reviewed in Ref. 22), suggesting that unfolding conditions dictate individual polypeptides to adopt distinct, highly variable aggregated structures (3, 4).

Here, molecular chaperones and differential centrifugation were used to address the degree of structural variation in different populations of homo-aggregates from non-pathogenic proteins. Depending on the denaturing conditions, proteins reproducibly formed very different aggregates, from small soluble chaperone-sensitive amorphous aggregates to more compact insoluble, chaperone-resistant aggregates. This is reminiscent of the PrPSC protein, which in mammals forms different strains of PrPSc, with distinct sensitivities to proteolytic enzymes, specific rates of infectivity, and different locations in the brain (23). Our results suggest that prions are not an exception.

**Different Proteins Can Form Hetero-aggregates**—Some cohesive aggregates have been suggested to derive from specific domain-swapping interactions (24, 25), implying that aggregation can take place only among identical or very similar polypeptides. Dissimilar co-aggregating polypeptides should therefore not interact with each other or inhibit, rather than accelerate, each other’s aggregations (26). Indeed, P22 tail spikes and coat proteins do not form hetero-aggregates (27).

However, here we show that cross-interactions between dissimilar co-aggregating proteins do take place and that they interfere with each other’s kinetics of aggregation and chaperone-recoverability. This is confirmed in vivo, whereby heterologous aggregation between huntingtin and p53 has been observed in mammalian cells (28). In Caenorhabditis elegans, expression of a spontaneously aggregating protein containing 82 poly-glutamine repeats (Q82), increased cytotoxicity, and sequestered otherwise soluble proteins into different types of prions (11, 35) as well as polyglutamine-rich aggregates in yeast (29, 30). Similarly, the spontaneous aggregation of the yeast prion [PIN(+)] induces the conversion of a number of soluble yeast proteins into different types of prions, suggesting a general mechanism of induced heterologous prion aggregation (30). One major implication of hetero-aggregation is that high cohesiveness in aggregates may not necessarily result from specific domain-swapping events but also from cooperative interactions between low-specificity sites, such as small hydrophobic patches and polar zippers on the surface of newly formed β-sheets (1, 31). Moreover, the intra-molecular rearrangement observed in the secondary structures of prionogenic, amyloidogenic, and common heat-induced aggregates are inconsistent with the high degree of structural preservation required for precise domain swapping (1).

**Aggregates Can Seed and Propagate Aggregation of Other Proteins**—Sub-stoichiometric amounts of one fast co-aggregating protein can significantly increase aggregation kinetics, oligomeric states, and chaperone-resistance properties of an otherwise soluble, slow-aggregating protein. This suggests that a conformational change, initially induced by heterologous MDH-G6PDH interactions, is further propagated to other G6PDH aggregates, without necessarily involving direct contacts with the initial MDH seed. This proteinaceous infectious behavior is similar to that of yeast prions, whereby a minority of one type of prion affects the onset and propagation kinetics of other yeast prions (30). We show here that non-pathogenic proteins may also generate under stress proteinaceous infectious aggregates.

Structurally fluid small aggregates formed by acid precipitation of non-pathogenic proteins are more toxic to mammalian cells than larger more stable amyloids (32). Likewise, prion seeding is more efficient when initiated with structurally fluid small oligomeric sup35 complexes, than with large “mature” particles (33). Similarly, we found that small soluble G6PDH aggregates formed at 52°C were more efficient seeds than insoluble large aggregates formed at 62°C (not shown). Hence, proteinaceous infectious behavior in non-pathogenic proteins may reach an optimum during the early phases of stress-denaturation in a mechanism that does not fundamentally differ from the “nucleation polymerization” model suggested for prion propagation (33, 34).

**Molecular Chaperones Can Prevent and Revert Seed-induced Aggregations**—In stressed cells, many sensitive proteins may alternate between native and partially unfolded states, which, depending on the conditions and the presence of seeds, may choose to aggregate or revert to the native state. We showed here that the stability of one protein may depend on the stability and aggregation-propensity of other unrelated proteins in the same cellular compartment, implying a new, unsuspected network of protein-protein interactions in the cell. Yet molecular chaperones can control such interactions by sequestering active seeds and aggregation-prone intermediates. Neutralization of catalytic-seeds is likely to be more powerful than direct prevention-of-aggregation of intermediates, the later depending on limited non-catalytic one-to-one interactions with the chaperones.

During prolonged stresses, once unfolding intermediates exceed the binding capacity of cellular chaperones, aggregation may prevail and disaggregating chaperones become essential to remove toxic seeds and intermediates. The involvement of molecular chaperones in aggregate detoxification has been demonstrated in prokaryotes and eukaryotes. Over-expression of Hsp100 and/or Hsp70 dissolves stress-induced aggregates in E. coli (11, 35) as well as polyglutamine-rich aggregates in yeast and animals (reviewed in Ref. 36). Moreover, cascade aggregations are observed when the expression of Hsp networks is low, such as during apoptosis (37) or aging (38). Our in vitro results demonstrate that as expected from seed-induced increase in aggregate size, the dependence on ClpB for efficient disaggregation increases. It is therefore important, especially for mammalian cells devoid of ClpB-like chaperones, to maintain aggregates small enough for effective scavenging by the Hsp70 chaperone system alone (12), or alternatively push aggregation to completion (34) and form large compact and therefore less-toxic structures, such as inclusion bodies and aggresomes (39).

**CONCLUSIONS**

Our results suggest that a network of molecular chaperones can play the role of an intracellular defense mechanism against protein pathogens. It can specifically recognize infectious particles, such as proteinaceous infectious seeds, and prevent their propagation among other proteins in the cell by passive sequestration or active disruption of the infectious agent. Moreover, we show that properties initially assigned to prions, such as multiple strains, infectivity, compactness, and resistance to chaperones, are also shared by regular proteins lacking polyglutamine motives, or associated pathologies. Yet mutant, amyloidogenic, and prion proteins may distinguish themselves from the other proteins in the cell by their ability to undergo
conformational rearrangements at or around physiological conditions (40) when HSPs are not abundant. It is moreover possible that mutant, poly-glutamine rich, amyloidogenic, and prionogenic proteins form more compact aggregates that naturally resist the chaperone and protease networks and consequently evolve detectable pathological aggregates during the lifetime of a given organism. Our results imply that environmental stresses may strongly influence the ability of cells to counteract the early onset pathologic protein aggregations.

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REFERENCES
1. Fink, A. L. (1998) Folding Des. 3, R9–23
2. Jaenicke, R. (1995) Philos. Trans. R. Soc. Lond. B. Biol. Sci. 348, 97–105
3. Khurana, R., Gillespie, J. R., Talapatra, A., Minert, L. J., Ionescu-Zanetti, C., Millett, I., and Fink, A. L. (2001) Biochemistry 40, 3525–3535
4. Diamant, S., Ben-Zvi, A. P., Bukau, B., and Goloubinoff, P. (2000) J. Biol. Chem. 275, 21107–21113
5. Perutz, M. F., Germeroth, L., Schneider-Mergener, J., and Bukau, B. (1997) EMBO J. 16, 1501–1507
6. Carrell, R. W., and Lomas, D. A. (1997) Lancet 349, 350–352
7. Carrell, R. W., and Lomas, D. A. (1997) Lancet 350, 134–138
8. Orr, H. T. (2001) Annu. Rev. Genet. 35, 53–68
9. Satyal, S. H., Schmid, E., Schmidt, A., and Perutz, M. F. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6763–6768
10. Diamant, S., Ben-Zvi, A. P., Bukau, B., and Goloubinoff, P. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7665–7668
11. Mogk, A., Tomoyasu, T., and Bukau, B. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13732–13737
12. Ben-Zvi, A. P., and Goloubinoff, P. (2001) J. Biol. Chem. 276, 20429–20434
13. Veinger, I., Diamant, S., Buchner, J., and Goloubinoff, P. (1998) J. Biol. Chem. 273, 11032–11037
14. Laufen, T., Mayer, M. P., Beisel, C., Klostermeier, D., Reinstein, J., and Fink, A. L. (2001) Biochemistry, in press
15. Wurz, S., Diamant, S., and Goloubinoff, P. (1998) Biochemistry 37, 9688–9694
16. Rudiger, S., Germeroth, L., Schneider-Mergener, J., and Bukau, B. (1997) EMBO J. 16, 1501–1507
17. Carrell, R. W., and Lomas, D. A. (1997) Lancet 349, 350–352
18. Lorimer, G. H. (1996) FEBS Lett. 384, 29–33
19. Prusiner, S. B., Burton, D. R., and Williamson, R. A. (2001) EMBO J. 20, 11032–11037
20. Diamant, S., Ben-Zvi, A. P., Bukau, B., and Goloubinoff, P. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7665–7668
21. Rudiger, S., Germeroth, L., Bohnke, M., and Eggstein, M. (2001) Nature 408, 511–514
22. Soti, C., and Csermely, P. (2000) Biogerontology 1, 225–233
23. Skovranek, S., Prasch, T., Steiner, M., and Fink, A. L. (2001) Science 293, 1371–1372
24. Borchsenius, A. S., Wegrzyk, R. D., Newnam, G. P., Inge-Vechtomov, S. G., and Fink, A. L. (2001) J. Biol. Chem. 276, 20429–20434
25. Satyal, S. H., Schmidt, A., and Perutz, M. F. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 530–534
26. Scopes, R. K., and Morgan, V. J. (1997) Biochim. Biophys. Acta 1336, 293–301
27. Scopes, R. K., and Morgan, V. J. (1997) Biochim. Biophys. Acta 1336, 293–301
28. Scopes, R. K., and Morgan, V. J. (1997) Biochim. Biophys. Acta 1336, 293–301
29. Scopes, R. K., and Morgan, V. J. (1997) Biochim. Biophys. Acta 1336, 293–301
30. Scopes, R. K., and Morgan, V. J. (1997) Biochim. Biophys. Acta 1336, 293–301
31. Scopes, R. K., and Morgan, V. J. (1997) Biochim. Biophys. Acta 1336, 293–301
32. Scopes, R. K., and Morgan, V. J. (1997) Biochim. Biophys. Acta 1336, 293–301
33. Scopes, R. K., and Morgan, V. J. (1997) Biochim. Biophys. Acta 1336, 293–301
34. Scopes, R. K., and Morgan, V. J. (1997) Biochim. Biophys. Acta 1336, 293–301
35. Scopes, R. K., and Morgan, V. J. (1997) Biochim. Biophys. Acta 1336, 293–301
36. Scopes, R. K., and Morgan, V. J. (1997) Biochim. Biophys. Acta 1336, 293–301
37. Scopes, R. K., and Morgan, V. J. (1997) Biochim. Biophys. Acta 1336, 293–301
38. Scopes, R. K., and Morgan, V. J. (1997) Biochim. Biophys. Acta 1336, 293–301
39. Scopes, R. K., and Morgan, V. J. (1997) Biochim. Biophys. Acta 1336, 293–301
40. Scopes, R. K., and Morgan, V. J. (1997) Biochim. Biophys. Acta 1336, 293–301