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REVIEW

Chaperones and Proteases

Cellular Fold-Controlling Factors of Proteins in Neurodegenerative Diseases and Aging

Marie-Pierre Hinault,[1] Anat Ben-Zvi,[2] and Pierre Goloubinoff*[*,1]

[1]DBMV, Faculty of Biology and Medicine, Lausanne University, CH-1015 Lausanne, Switzerland; and [2]Rice Institute for Biomedical Research, Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL

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Abstract

The formation of toxic protein aggregates is a common denominator to many neurodegenerative diseases and aging. Accumulation of toxic, possibly infectious protein aggregates induces a cascade of events, such as excessive inflammation, the production of reactive oxygen species, apoptosis and neuronal loss. A network of highly conserved molecular chaperones and of chaperone-related proteases controls the fold-quality of proteins in the cell. Most molecular chaperones can passively prevent protein aggregation by binding misfolding intermediates. Some molecular chaperones and chaperone-related proteases, such as the proteasome, can also hydrolyse ATP to forcefully convert stable harmful protein aggregates into harmless natively refoldable, or protease-degradable, polypeptides. Molecular chaperones and chaperone-related proteases thus control the delicate balance between natively folded functional proteins and aggregation-prone misfolded proteins, which may form during the lifetime and lead to cell death. Abundant data now point at the molecular chaperones and the proteases as major clearance mechanisms to remove toxic protein aggregates from cells, delaying the onset and the outcome of protein-misfolding diseases. Therapeutic approaches include treatments and drugs that can specifically induce and sustain a strong chaperone and protease activity in cells and tissues prone to toxic protein aggregations.

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Index Entries: Proteasome; heat shock proteins; Hsp70; Hsp90; Hsp27; NSAIDs; inflammation; aggresome; fever.

The Folding Pathway Leading to Native and "Alter-Native" States

The polypeptide primary sequence contains all of the necessary information for it to reach a native three-dimensional structure, without the apparent need for external factors (Anfinsen, 1973). During *de novo* synthesis, or translocation into the mitochondria, proteins emerge unfolded from the ribosome or the translocation pores. When exposed to water, hydrophobic segments tend to spontaneously collapse into water-excluding hydrophobic cores, surrounded by hydrophilic residues (Morgan et al., 1998). The spontaneous formation of secondary structures therein may then lead to a discrete native structure (Fig. 1, reaction 1), which is, in principle, more stable than the unfolded state. A natively folded monomer may already be fully functional, as in the case of myoglobin. Yet, evolutionally more complex enzymes may form discrete native oligomers composed of several near-native monomers, as in the case of the hemoglobin tetramer (Suzuki and Imai, 1998). Although very similar to functional myoglobin, the unassembled nonfunctional hemoglobin

*Author to whom all correspondence and reprint requests should be addressed. E-mail: Pierre.Goloubinoff@unil.ch

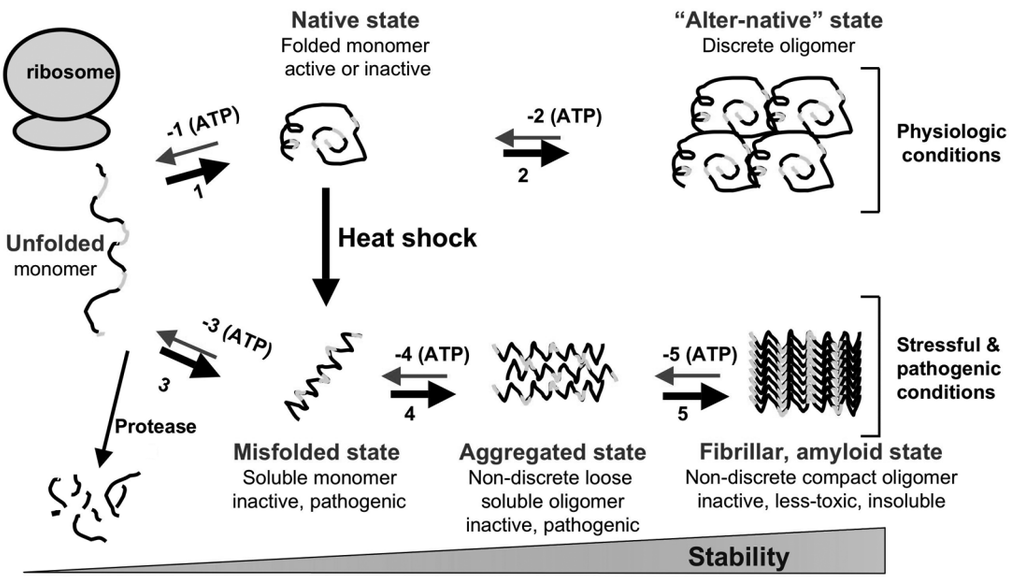

Fig. 1. Model for the role of molecular chaperones in protein unfolding. Under physiological conditions (upper path), an unfolded newly synthetised, or translocated polypeptide may spontaneously *fold* (1) into a native monomer, and/or (2) further assemble into an "alter-native" discrete oligomer. ATPase unfolding chaperones can actively deoligomerize (-2) "alter-native" oligomers into monomers and further unfold (-1) near-native monomers to be translocated across membranes or degraded. Under stressful conditions (lower path), or because of mutations, a newly synthesized may spontaneously *misfold* (3) into a soluble, possibly toxic monomer, which may further assemble (4) into a continuum of small toxic oligomers that may further condense (5) into less toxic, compact amyloids. Specific organic molecules, may destabilize, and ATPase chaperones, actively scavenge (-5, -4) compact amyloids and fibbers into looser, albeit more toxic oligomers. ATPase chaperones may further unfold (-3) and detoxify misfolded monomers into harmless protease products or natively refolded proteins. The leftward reactions are against entropy and ATP hydrolysis may be needed for the chaperones and proteases to forcefully unfold stably misfolded or alternatively folded protein structures.

monomer is in a near-native state, which is, in contrast, prone to degradation by proteases. The ability of folded proteins to alternate between native, near-native or "alter-native" states (Fig 1, upper path), demonstrates that within the native folding pathway, a single polypeptide chain may reach several distinct native states, separated only by shallow kinetic barriers (Fig. 1, reaction 2). Thus, to regulate a given activity, cells may modulate the equilibrium between an active native state and an inactive "alter-native" state of a given polypeptide, instead of more costly modulations of protein amounts by balancing synthesis with degradation. However, this energetically more favourable level of control necessitates the presence of fold-controlling factors that can specifically distinguish between native, "alter-native" and nonnative states of thousands of different proteins in the cell. Experimental data now point at the molecular chaperones, in particular at Hsp90 and Hsp70, as being such fold-controlling factors that can regulate transitions between native monomeric and oligomeric states and thus regulate the activity of a plethora of native protein functions in the cell (for a review about Hsp90, see Pratt and Toft, 2003).

The Misfolding Pathway Leading to Aggregation

Although the polypeptide primary sequence may contain all of the necessary information to reach a native three-dimensional structure (Anfinsen, 1973), under physiological conditions of temperatures, molecular crowding and when the concentration of un/folding intermediates is high, mistakes in the folding/refolding process may occur, leading to aggregation (Ellis, 2001). Some native proteins are intrinsically more stable than others. Destabilizing mutations, the presence of degradation or secretory signals, transit-peptides or tags (SsrA or ubiquitin), can increase the rate of spontaneous unfolding and the conversion of native proteins into protease-sensitive

or translocation-competent polypeptides (Prakash and Matouschek, 2004).

Conceptually, protein *misfolding* is to be clearly distinguished from *unfolding* and *aggregation*. Unfolding is the partial, or global, loss of tertiary and secondary structures in a protein. Misfolding addresses possible intramolecular rearrangements that may take place during synthesis, or following stress-induced unfolding, within the tertiary and secondary structures of a given polypeptide, as compared to its native structure. The propensity of a protein to misfold depends on intrinsic properties, such as the relative amount and distribution of hydrophobic and charged residues (Chiti et al., 2002), and on the length of the polypeptide (Mogk et al., 1999; Uversky, 2002). In contrast, aggregation is a concentration-dependent, intermolecular process, whereby already formed misfolded monomers tend to associate mostly by hydrophobic interactions in a highly cooperative manner.

Noticeably, a minority of proteins, such as Tau and α-synuclein in neural cells, Late Embryogenesis Abundant (LEA) proteins, and dehydrins in plant seeds are "natively unfolded" proteins that are inclined to stay naturally devoid of secondary structures in the cytoplasm under normal conditions (Uversky, 2002). In contrast, the remaining majority of proteins characterized by an average charge-to-hydrophobicity ratio are highly unstable when partially unfolded in the cell. Depending on the degree and duration of an unfolding stress, they tend to readily acquire nonnative misfolded structures enriched in β-sheets that gradually aggregate into larger, more compact and less soluble complexes (Fig. 1, reactions 3–5). Hence, in the misfolding pathway, the protein structure is much less controlled by the primary sequence than in the native folding pathway. Misfolding intermediates spontaneously accumulate short default cross β-conformations, some with hydrophobic surfaces, at the expense of more labile native α-helices (Uversky, 2003). Although proteins in their misfolded state must be thermodynamically less stable than in their native state, stable ensembles of misfolded proteins can accumulate and affect cells because exposed hydrophobic surfaces must cooperatively associate to escape direct contact with water and thus form highly cohesive aggregates, fibrils, and amyloids. Here, unlike with discrete native oligomers, aggregates span a wide range of sizes and morphologies, depending on the concentration of the misfolded monomers, the presence of mutations, or on the intensity and duration of a stress applied (for a review, *see* Jahn and Radford, 2005). Compared to native proteins, aggregates may be detrimental to the cell, as they are devoid of biological activity in the classical sense. Large aggregates can physically disrupt cells and tissues. They expose hydrophobic surfaces that can interfere with other misfolding proteins and with membranes. Disturbance of membrane function is of particular importance to neurons because minor alterations causing ion leakage (Lashuel et al., 2002), may have dramatic consequences on neural activity (for a review, *see* Caughey and Lansbury 2003). Moreover, misfolding protein intermediates may interfere with the native folding pathway of other proteins in the cell and induce cross-aggregations (Satyal et al., 2000; Ben-Zvi and Goloubinoff, 2002; Gidalevitz, et al., 2006), a proteinaceous infectious behavior, which is particularly effective in the case of mammalian and yeast prions (Prusiner, 1989; Jones and Tuite, 2005).

The Cellular Role of Molecular Chaperones

Molecular chaperones are composed of several classes of conserved proteins (Table 1) with essential physiological functions such as assisting the folding of nascent polypeptides and pulling proteins across membranes (Neupert and Brunner, 2002; Deuerling and Bukau, 2004; De Los Rios et al., 2006). In addition, most molecular chaperones are stressed-induced proteins (Hsps) that can prevent the aggregation and facilitate the correct refolding of stress-labile, or mutation-sensitive proteins (Hartl and Hayer-Hartl, 2002). Some chaperones, in particular the small Hsps, can also protect membrane from environmental stresses (Torok et al., 1997). By virtue of their strong buffering on the deleterious effects of mild aggregation-inducing mutations, the molecular chaperones serve as capacitors for phenotypic variations, modulating the pace of molecular evolution (Queitsch et al., 2002).

The stress-inducible nature of many molecular chaperones has led to their early classification among the heat shock proteins (Hsps) according to their apparent molecular weights in gels: Hsp100, Hsp90, Hsp70 (Hsp40, Hsp20), Hsp60 (Hsp10), and Hsp22/27 in eukaryotes (co-chaperones in brackets), corresponding in *Escherichia coli* to ClpB, HtpG, DnaK (DnaJ, GrpE), GroEL (GroES), IbpA/B, respectively (Table 1). Various mechanisms by which different individual molecular chaperones may assist the native folding of proteins have been studied

Table 1
Major Classes of Molecular Chaperones In Mammals: Cellular Location and Central Functions in Protein Misfolding Diseases

Name		Cellular location	ATPase	Passive prevention of aggregation	Active unfolding	Signalling & prevention of apoptosis (overexpression)
Eukaryotes	Prokaryotes	Eukaryotes				
Hsp100	Clp B	MT	+	–	+	?
Hsp90	HtpG	Cyt, ER, MT	+	+	?	+
Hsp70, Hsc70	DnaK	Cyt, ER, MT	+	+	+	+
Hsp40	DnaJ	Cyt, ER, MT	– (catalyzes ATPase in Hsp70)	+	–	+
Hsp60	GroEL	MT	+	+	+	–
Hsp22/27	IbpA, IbpB	Cyt, ER, MT	–	+	–	+

Cyt, cytoplasm; ER, endoplasmic reticulum; MT, mitochondria; Hsp, heat-shock protein.

in vivo using mutagenesis and genetic approaches, and in vitro using biochemical, biophysical and even physical approaches (Goloubinoff et al., 1989; Goloubinoff et al., 1999; Ben-Zvi et al., 2004; De Los Rios et al., 2006). Different chaperones display mutually nonexclusive properties. Some "binding" chaperones, such as Hsp90, Hsp70, Hsp60, Hsp40, and Hsp22/27, can provide adhesive surfaces which, upon interaction with partially denatured polypeptides, can passively reduce the degree of aggregation (Chatellier et al., 2000; Mogk et al., 1999). "Folding" chaperones, such as Hsp100, Hsp70, and Hsp60 (possibly also Hsp90), are involved in the ATP-dependent native refolding of artificially denatured polypeptides (Table 1) (Goloubinoff et al., 1989; Goloubinoff et al., 1999; for a review, *see* Ben-Zvi and Goloubinoff, 2001). Misfolded polypeptides may be transferred from "binding" to "unfolding" chaperones, thereby allowing optimal cooperation in the recovery of native proteins by the various chaperone systems (Veinger et al., 1998; Ben-Zvi and Goloubinoff, 2001; Mogk et al., 2003).

The remarkable ability of the disaggregating chaperones Hsp70 and Hsp100 to recognize, bind misfolded and aggregated proteins, and forcefully convert the latter into natively refoldable polypeptides demonstrates that chaperones can act as true enzymes. Like enzymes, they accelerate the conversion of stable, high-affinity misfolded substrates into more stable, low-affinity natively refolded products. Some chaperones can use the energy of ATP-hydrolysis to overcome the high kinetic barrier between the two states (Fig 1, reactions -5, -4, -3) (Goloubinoff et al., 1999; Ben-Zvi et al., 2004). Understanding the mechanisms by which the chaperone and protease network can specifically recognize toxic misfolded protein conformers in the cell and convert them into nontoxic, natively refolded or degraded polypeptides is central to the comprehension of protein-misfolding neuropathologies and for conceiving possible therapies.

The Role of the Molecular Chaperones in Neurodegenerative Diseases

The accumulation in and outside neurons, of protein aggregates compacted into amyloid plaques, fibrils or neurofibrillary tangles, which is accompanied by an excessive inflammatory response, oxidative stress, and cell death, are common characteristics of most neurodegenerative diseases, such as Alzheimer's, Parkinson's, Huntington's diseases, amyotrophic lateral sclerosis, and prion encephalopathies (Dobson, 1999; Muchowski and Wacker, 2005). Age is the main risk factor in most protein-conformation neurodegenerative diseases. Moreover, several protein conformational disorders may develop in the same aging patients, suggesting a general failure of the protein fold-quality control in aging tissues (Hamilton and Bowser 2004; Forman et al., 2002; Soti and Csermely, 2003).

There is now a large body of evidence indicating that the molecular chaperones and chaperone-controlled proteases, such as the proteasome, belong to a cellular network that can prevent and reduce the formation of toxic aggregates and possibly

eliminate already formed toxic protein aggregates in neurodegenerative diseases (Muchowski et al., 2000; Barral et al., 2004; Klucken et al., 2004; for a review, *see* Muchowski and Wacker, 2005).

Most reports thus far correlate between the presence of one, or a selective choice of molecular chaperones (typically Hsp70, Hsp40, Hsp90, and/or Hsp27) with reduced amounts of a given neurotoxic aggregate and, more rarely, with the clearance of pre-existing aggregates. The general picture is as follows.

1. Overexpression of one, or better, of the whole chaperone and protease network strongly correlates with a diminution of protein aggregation, toxicity, inflammation, and neuronal loss. Overexpression of Hsp70 inhibits inflammation, the production of reactive oxygen species (ROS) and consequent apoptosis.
2. Underexpression of members of the chaperone or the protease network, as in aging, by RNA inhibition, mutations or by using specific inhibitors, strongly increases toxic protein aggregation, inflammation and cell death.
3. Molecular chaperones act ubiquitously. No particular chaperone can be assigned to the relieving or curing of a specific protein conformation disorder. All protein-misfolding diseases, among them neurodegenerative ones, favourably respond to chaperone overexpression and negatively respond to chaperone underexpression, mutation or inhibition.
4. Seldom, imbalanced expression between the various chaperones (typically Hsp70 and co-chaperones) can lead to deleterious effects.

Below, we have summarized some milestone observations about the central role of molecular chaperones as factors that control the fold-quality of proteins in the cell, in the particular context of neurodegenerative diseases.

Alzheimer disease (AD) is characterized by the extra-cellular accumulation of Aβ-amyloids and the intracellular accumulation of neurofibrillary tangles of the otherwise natively unfolded Tau protein (for a review, see LaFerla and Oddo, 2005). Direct interactions among Hsp70, Hsp90, and Tau have been observed in COS-1 cells expressing human Tau. Induction of Hsp70 and Hsp90 by mild poisoning with geldanamycin, correlated with increased Tau solubility, whereas Hsp70 and Hsp90 suppression by RNA interference significantly decreased Tau solubility (Dou et al., 2003).

Parkinson's disease (PD) is characterized by intracellular and membrane-interfering aggregates of the presynaptic neuronal protein: α-synuclein (Tofaris et al., 2005). Whereas expression of α-synuclein in *Drosophila* flies leads to neuronal loss, its co-expression with human Hsp70 significantly decreased neuronal loss (Auluck et al., 2002). This strongly suggests that human Hsp70 can reduce neurotoxicity by actively unfolding α-synuclein aggregates. In mammalian cells, expression of Hsp27 has protective effects against α-synuclein and huntingtin-induced cell-death (Zourlidou et al., 2004), probably by passively sequestering the toxic interactive surfaces of the aggregates.

Familial amyotrophic lateral sclerosis is a neurodegenerative disease associated with mutation-induced aggregation of Cu/Zn superoxide dismutase (SOD-1) (Selverstone Valentine et al., 2005). Hsp70 co-expression with aggregation-prone SOD-1 can significantly lower SOD-1 aggregation and prolong cell viability (Bruening et al., 1999). A direct association of Hsp70, Hsp40, and αB-crystallin with mutant SOD-1 was demonstrated by coimmunoprecipitation. In human cell lines, a direct association between human mutant SOD-1 and Hsp70, Hsp40 and α–crystallin has been shown (Shinder et al., 2001).

Neuronal loss from various polyglutamine diseases results from mutations in different proteins that generate in-frame expansions of glutamine repeats (polyQ tracks). In a cellular model for Huntington's disease, human neuroblastoma cells expressing huntingtin (with extended polyQ tracts), and the co-expression of both Hsp70 and Hsp40 or of only Hsp40, significantly lowered huntingtin aggregation and cell death (Wyttenbach *et al.*, 2000). Hsp40 is the central co-chaperone of Hsp70 that can bind aggregates by itself, as well as catalyze Hsp70 binding onto the aggregate (Table 1) (Laufen et al., 1999; Ben-Zvi et al., 2004). Thus, excess Hsp40 may sequester toxic conformers. Similarly, the co-expression in mouse of the yeast chaperone Hsp104 with the first 171 residues of a huntingtin mutant resulted in a decrease of aggregate formation and in a 20% increased survival as compared to mice expressing only the mutant huntingtin (Vacher et al., 2005). Hsp27 expression was also shown to significantly inhibit polyQ-induced cell death, although without reducing the formation of detectable aggregates (Wyttenbach et al., 2002). As with excess of Hsp40, passive binding of toxic hydrophobic surfaces by Hsp27 may suffice to prevent the onset of an apoptotic signal. Moreover, indicating that the protective mechanism mediated by Hsp27 differs from active aggregate-scavenging by Hsp70/Hsp40, some endogenous Hsp70 and Hsp40, but not Hsp27, were found sequestered into the insoluble protein

inclusions. In this case, alongside with neutralizing toxic soluble aggregates in the cytosol and passively preventing the formation of hydrophobic pore-forming neurotoxic aggregates (Lashuel et al., 2002), small Hsps may also stabilize aggregate-damaged membranes (Torok et al., 2001).

In a cellular model of spinocerebellar ataxias, Hsc70 and two Hsp40 isoforms were colocalized with polyQ aggregates of mutant ataxin-3 fragments. Coexpression of human Hsp40 suppressed ataxin-3 aggregates, whereas coexpression of Hsp70 or Hsp27 remained ineffective (Chai et al., 1999). Expression of another human Hsp40, Hdj-2, could also suppress aggregation of mutant androgen receptor and of ataxin-1 (Cummings et al., 1998; Stenoien et al., 1999). In a system modeling spinal and bulbar muscular atrophy—cultured neuronal cells expressing truncated androgen receptor protein with an expanded polyQ tract—only the combination of overexpressed Hsp70 and Hsp40 can reduce the formation of aggregates and prevent apoptosis, whereas over expressing Hsp40 alone has no effect (Kobayashi et al., 2000).

Prion related-diseases are particular in that the toxic protein aggregates can also propagate and induce neighbouring cells to produce toxic protein aggregates (Prusiner, 1989) and undergo apoptosis. Prion disease in mammals results from the conformational conversion of a native, α–helix rich PrP^{c} protein, into an aggregation-prone, β-sheet-enriched, infectious PrP^{sc} species. In yeast, several nonpathological prions have been identified, from proteins such as Sup35, Ure2, and Rnq1 (Jones and Tuite, 2005). Overproduction of the ATPase chaperone Hsp104 (the yeast homologue of bacterial ClpB), or of a cytosolic Hsp70 (Ssb) can transiently cure the [PSI+] yeast prion phenotype of the Sup35 protein, suggesting that disaggregating molecular chaperones can act upon prion particles (Chernoff et al., 1995). The role of Hsp70 chaperones in prion curing is, however, to be considered cautiously as overproduction of another member of the Hsp70 family, Ssa1, resulted in the propagation of the yeast prion (Chernoff et al., 1999).

Possible Detrimental Effects of Chaperone Activity

Strong evidence points at abnormally exposed hydrophobic surfaces as being the pathogenic and infectious parts of the protein aggregates (Bieschke et al., 2004). Simply because of differences in the surface/volume ratio, the *specific* pathogenicity and infectiousness of a given misfolded protein is expected to be the highest when it is in a least-compacted monomeric state, and the lowest when it is assembled within large compact aggregates (Fig. 1, reactions -5 and -4). Eukaryotic cells have developed a dynein- and microtubule-dependent transport mechanism, named the aggresome, which can actively concentrate small toxic aggregates into denser, presumably less harmful inclusion bodies (Kopito, 2000). This raises the possibility of a conflict between aggregate-detoxification by aggresome-mediated compaction (Fig. 1, reaction 6) vs detoxification by chaperone-mediated unfolding of aggregates (Fig. 1, reactions -5, -4, -3), the latter being able to transiently generate smaller but more toxic forms from larger less toxic amyloids. Increased infectiousness has been demonstrated in vitro with sonication treatments which, mimicking scavenging chaperones, artificially converted large noninfectious PrP^{sc} particles into smaller infectious ones (Bieschke et al., 2004). In yeast, although overexpression of the scavenging chaperone Hsp104 can virtually cure the [PSI+] prion phenotype, deletion of the *hsp104* gene inhibits [PSI+] propagation (Chernoff et al., 1995). This confirms that under specific conditions, such as during "unprotected" scavenging by Hsp104 or Hsp70, some toxic aggregates may form and prions may propagate from larger inactive forms of aggregates.

Although there is overwhelming evidence that Hsp70 overexpression, prior to or during early neurotoxic protein aggregation, has powerful neuroprotective and anti-apoptotic effects (Magrane et al., 2004), occasional reports indicate that Hsp70 overexpression may accelerate cell death, as in the case of tumor necrosis factor (TNF)-treated Cos-1 cells (Ran et al., 2004) or of lentiviral-mediated overexpression of Hsp70, alongside α-synuclein in rat brains (P. Aebischer, personal communication). Interestingly, Hsp70 has a unique molecular mechanism whereby the energy of ATP is used to recruit random movements of the chaperone molecule to apply a local unfolding force on the misfolded polypeptide susbstrate (Ben-Zvi et al., 2004; De Los Rios et al., 2006). Thus, individual Hsp70 molecules fully suffice to breakdown large aggregated particles into smaller ones. However, the cooperativity of at least three Hsp70 molecules, independently bound to the same misfolded polypeptide, is necessary to complete the unfolding of the latter into a natively refoldable species (Ben-Zvi et al., 2004). This implies that a mild but insufficient expression of Hsp70 in the

cell, as in the neuronal tissues of aging mammals, may suffice to initiate partial breakdown of some least-harmful amyloids into fragmented, more toxic particles. However, at this stage, the reaction could become stalled for lack of sufficient Hsp70 molecules to cooperate in the final conversion of most toxic species into harmless natively refoldable, or protease-degradable, species (De Los Rios et al., 2006). There is no obvious Hsp104 homolog in human that can assist Hsp70-mediated protein disaggregation. However, neural cells may still recruit binding chaperones, such as Hsp27 (Patel et al., 2005), to reduce the transient toxicity of newly formed hydrophobic surfaces during amyloid scavenging by Hsp70.

The Role of the Proteasome in Neurodegenerative Diseases

In agreement with the observations that chaperones and the proteasome carry complementing protective cellular mechanisms against protein-conformational diseases (Fig. 1, reaction 7), immunostaining has revealed the presence of ubiquitin, proteasomal subunits and of Hsp70 in neuronal nuclei of cells with amyloids of several diseases, including AD, Parkinson's disease, and prion diseases (Adori et al., 2005). Inhibition of the proteasome activity with MG-132 in mouse cell lines and primary neurons, or inhibition of calpain or other cytosolic proteases, favors the cytosolic accumulation of PrP^{sc} and prion pathogenesis (Wang et al., 2005c). Noticeably, the endoplasmic reticulum Hsp70 (BIP) controls the folding of PrP, and an anomalous, prolonged association between BIP and mutant PrP32 inhibits subsequent proteasome-mediated degradation of the misfolded PrP32, leading to prion disease (Jin et al., 2000). Similarly, aggregated α-synuclein strongly associates and inhibits proteasome activity, suggesting that proteasome-mediated degradation of misfolded α-synuclein is a central cytoprotective mechanism in Parkinson's disease. Indeed, rats treated with proteasome inhibitors developed typical Parkinson pathologies (Nomoto and Nagai, 2005). In human neuroblastoma cells expressing the α-synuclein mutant A53T, co-expression of Parkin, a E3-ubiquitin-ligase, alleviates the α-synuclein toxicity (Petrucelli et al., 2002). Similarly, in human neuroglioma cells, co-expression of CHIP, an Hsp70 co-chaperone with an E3-ubiquitin-ligase activity acting as a molecular switch between proteasomal and lysosomal degradation pathways, decreases the levels of aggregated α-synuclein (Shin et al., 2005). In COS cells, CHIP co-expression with a truncated polyQ-rich human huntingtin suppressed both huntingtin aggregation and toxicity (Miller et al., 2005). In mouse neuroblastoma cell lines, overexpression of another E3 ubiquitin ligase, Dorfin, provided protection from neurotoxic mutant SOD-1 (Niwa et al., 2002). Because E3 ubiquitin-ligases have a significantly more restricted spectrum of substrates than the molecular chaperones and proteases, it is possible that targeted degradation of specific neurotoxic aggregates can be achieved by overexpressing specific E3 ubiquitin-ligases.

Aging

A general age-dependent decrease in the ability to express Hsps and antioxidant enzymes under stress is observed in all multicellular organisms, from mammals, nematodes, and insects, to plants (Morrison et al., 2005; Starnes et al., 2005). Failure of aging humans to induce and maintain a molecular heat-shock response consequent to an abiotic, or a cellular stress, strongly correlates with the onset of protein-conformation disorders, in particular of neurodegenerative diseases (Soti and Csermely, 2003). Figure 2A shows a scheme of the various age-dependent processes, correlating the decline of chaperone and proteasome activity in the cell, with expected levels of toxic aggregates and of inflammation leading to fatal neuronal loss. Because the stability of the native state is primarily an intrinsic property of each protein, the rate of spontaneous protein unfolding, leading to misfolding and aggregation, is expected to be rather constant during lifetime, with a possible mild increase later in life due to cumulative mutations and damages from polluting chemicals such as heavy metals (Basha et al., 2005), ionizing radiations, ozone, tobacco smoke, and environmental stresses (ultraviolet light, temperature variations etc.) (Manton et al., 2004; Landrigan et al., 2005). Early in life, however, the chaperone and protease network is optimally inducible and can effectively prevent the steady formation of small toxic aggregates and, furthermore, actively unfold, reactivate, or eliminate all the misfolded species that may form in young unstressed cells. Later in life, when the chaperone and protease network becomes gradually deficient (Heydari et al., 2000), toxic aggregates may accumulate, but partial protection may still be achieved by active secretion, lysosomal degradation, or aggresome-mediated sequestration of the most toxic species into dense fibbers and compact amyloids (Kopito, 2000). Yet, above a critical

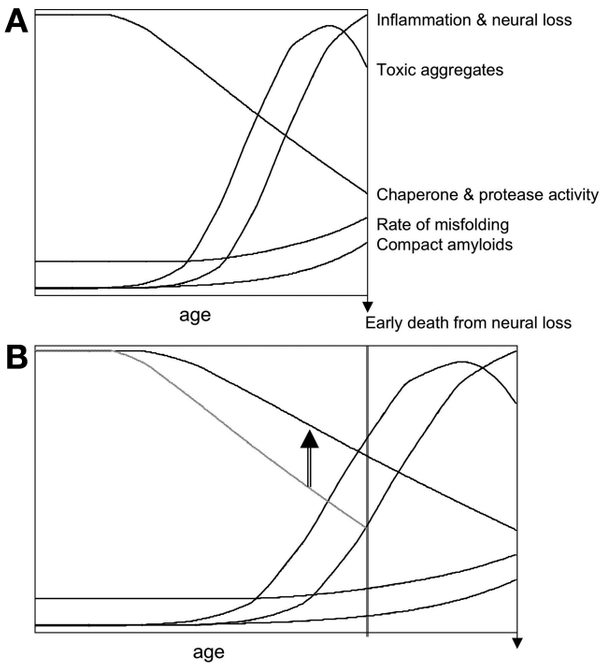

Fig. 2. Scheme of the age-dependent processes leading to fatal neuronal loss. Early in life, the chaperone and protease networks are optimally inducible and active. They effectively prevent the accumulation of toxic protein aggregates. The rate of spontaneous misfolding and aggregation is constant, with a possible mild increase later in life because of cumulative mutations and stress-induced damages. Later in life, the chaperone and protease network become deficient and toxic aggregates accumulate. Some aggregates are partially sequestered by the aggresome, others induce inflammation and reactive oxygen species, leading to fatal neuronal loss. **(A)** Early failure of the chaperone and protease network lead to early death by neurodegeneration. **(B)** Effect of improved activity (arrow) of the chaperone and protease network, by Hsp-inducing drugs or treatments, delay in fatal neuronal loss and increase in life expectancy.

threshold of aggregation, the various cell defences may become overpowered, inducing an inflammatory response with the release of ROS, leading to apoptosis (Soti and Csermely, 2003).

The Proteasome and Aging

The activity of the proteasome has been reported to decrease with age in cell cultures and in vivo (for a review, *see* Gaczynska et al., 2001). Decline of proteasome activity has been reported in several aged human tissues, such as lens, muscle, lymphocytes, and epidermis, as well as in senescing human primary cultures. A similar tendency was observed in aging tissues of other mammals (for a review *see* Chondrogianni and Gonos 2005). Free ROS produced by chronic inflammation can cause cumulative damages to cellular macromolecules and in particular lipids and proteins, and seems to contribute to senescence and aging, age-related disorders, and neuromuscular degenerative diseases. The accumulation of oxidized proteins has been reported in many aging and models of protein-misfolding diseases. In young individuals, mildly oxidized and aggregated proteins are rapidly degraded by the proteasome. As in the case of molecular chaperones, the specificity of the proteasome could depend on the extent of misfolding in oxidized proteins. The 20S proteasome may directly bind and degrade such mildly damaged proteins, without needing the ubiquitin targeting. Severely oxidized, aggregated, and crosslinked proteins, however, are less efficient substrates for degradation and may stall the proteasome during aging and in many age-related conformational disorders (Ferrington et al., 2005; Davies and Shringarpure, 2005). During cellular aging and inflammatory degenerative diseases, the combination between the steady increase of protein damage from ROS and the steady decrease in proteasome expression and activity may result in the accumulation of toxic protein species, leading to cell death (for a review, *see* Ciechanover, 2006).

The artificial triggering of a heat-shock response in neural cells during the onset of neurodegenerative diseases, or during aging, is expected to improve cellular levels of chaperones and proteases (Fig. 2B, arrow) and consequently delay the accumulation of toxic protein aggregates. To lower neuronal loss and increase life quality and expectancy, therapeutic approaches may include the use of Hsps inducers, in turn preventing and actively reverting protein aggregation. Complementing therapeutic approaches may include the use of direct inhibitors of protein aggregation and of anti-oxidants able to directly quench ROS damages, and inhibit ROS-mediated apoptotic signals.

Therapeutic Approaches: Aggregate-Interfering Compounds

A number of small organic molecules have been shown to retard and, more rarely, to reverse the formation of toxic protein aggregates in various in vivo and in vitro model systems. Compounds with high affinity for aggregated proteins, such as the

amphiphilic fluorescent dye Bis-ANS, can inhibit the in vitro aggregation of PrP^{sc} (Cordeiro et al., 2004). Similarly, congo red, a dye used to detect amyloid deposits in histological slices, can inhibit α-synuclein formation in vitro and in cell cultures (Heiser et al., 2000). Feeding congo red or cystamine to *Drosophila* flies expressing a polypeptide with an expanded polyQ-repeat suppressed degeneration of photoreceptors and neurons (Apostol et al., 2003). In a transgenic mouse model for Huntington disease, the transglutaminase inhibitor cystamine, increased survival and improved motor function (Dedeoglu et al., 2002). Rifampicin applied to rat cells inhibited the aggregation and neurotoxicity of synthetic Amyloid-β peptides (Tomiyama et al., 1996). Interestingly, rifampicin can strongly bind to α-synuclein monomers, inhibit fibrillation, and possibly mediate the solubilization of preformed α-synuclein fibbers in vitro, suggesting a direct interference of rifampicin with toxic α-synuclein aggregates in the cell (Li et al., 2004b). Similarly, curcumin treatments abrogated retention of myelin protein zero aggregates into the endoplamic reticulum (ER) and reduced aggregate-induced apoptosis (Khajavi et al., 2005), as well as the formation of Amyloid-β oligomers and fibrils (Yang et al., 2005).

The various beneficial effects of small organic compounds, such as Bis-ANS, Thyoflavin-T, curcumin, and congo Red, on aggregate reduction in cells raises the question of their precise mechanism of action. In a cell-free system, interfering compounds often bind strongly to stable aggregates, although without causing solubilization. Therefore, similar high-affinity binding of such compounds in the cell may not necessarily drive to the solubilization of compact amyloids. Consequently, candidate therapeutic molecules should first be tested for their potential ability to induce the expression of molecular chaperones and proteases. This should allow one to distinguish between direct aggregate-interfering compounds and Hsp-inducing compounds acting indirectly on aggregates by way of increasing the aggregate- scavenging activity of the cell.

Hsp-Inducing Treatments

The demonstrated ability of stress-inducible chaperones and proteases to prevent the accumulation of toxic protein aggregates in the cell implies that drugs capable of increasing Hsp expression, without increasing the misfolding propensity of sensitive proteins, may delay the onset of, and possibly cure, protein-misfolding and age-related diseases.

Caloric Restriction and Hormonal Treatments

In mammalian cells, glucose starvation induces the massive accumulation of a specific subset of ER-located molecular chaperones, such as Grp78 and Grp94 (for a review, *see* Lee, 2001). Grp78 overexpression provides protection of cells against induced apoptosis (Liu et al., 1997; Reddy et al., 1999). Interestingly, high levels of both chaperones in the brain of young rats correlate with higher resistance to seizures, compared to older rats expressing less Grp78 and Grp94 (Little et al., 1996). Mild hypoglycemia may thus suffice to induce ER chaperones, which in turn may increase the resistance of cells to various stresses, age-dependent protein aggregations in the ER, and aggregate-induced apoptosis. Noticeably, caloric restriction is the most effective anti-aging treatment in mammals (for a review, *see* Kirkwood and Shanley, 2005). Caloric restriction, while increasing levels of molecular chaperones, also prevents age-related declines of glycolytic enzymes in the neural retina of aging rats (Li et al., 2004a). Interestingly, treatments with estrogens and androgens, in addition to glucose regulating hormones, also increased Hsp70 levels in human neurons and induced resistance to toxic intracellular amyloids (Zhang et al., 2004). It is therefore possible that the anti-aging effect of mild caloric restriction and of some hormonal treatments affecting glucose uptake are mediated, in part, by way of induced Hsp-expression with anti-inflammatory, anti-apoptotic, and anti-protein aggregation activities.

Heat-Shock

Since antiquity, repeated mild heat-shock treatments, in the form of hot baths and saunas, are considered to have age-retarding and relieving effects on age-related chronic diseases. It is now well established in all organisms that repeated mild thermal treatments can prime the accumulation of heat-inducible proteins, most of which being transcription factors, molecular chaperones and proteases (Kregel, 2002). Indeed, mild heat-shock treatments are the simplest way to induce Hsps in the cell and gain protection, both against direct damages from subsequent harsher environmental stresses, and against indirect damages from ROS-induced inflammation leading to cell death (Beere, 2004).

The Role of Fever

Hippocrates (460–377 B.C.) is quoted to have said: "Give me the power to create a fever and I shall cure every illness." During inflammation, homeothermal organisms develop high fever, during which high levels of Hsps accumulate everywhere, including in the central nervous system, in correlation with the onset of various healing processes (for a review, *see* Moseley, 1998). As in the case of the response to heat-shock, the ability of animals to induce high fever and Hsps decreases with age (Soti and Csermely, 2003; Macario and Conway de Macario, 2005). Elevated levels of heat-shock proteins are nonetheless often observed associated to various forms of amyloids and protein aggregates in degenerated neural tissues of deceased aged patients, which is not necessarily in contradiction with the general observation that Hsp-inducibility late in life is mediocre: aging tissues exposed to toxic aggregates may have first attempted to recruit some Hsps, but having failed to recruit enough of them, succumb to programmed cell death. Noticeably, repeated mild heat-shock treatments can partially restore Hsp-inducibility in aging flies (Hercus et al., 2003) and increase post-heat stress survival of organisms as different as bacteria, yeast, plants, nematodes, and mammals (Rattan, 2004). This suggests that repeated mild heat-treatments in aging humans with protein conformational diseases may restore some ability to induce and accumulate molecular chaperones and proteases, and thus prevent toxic aggregation in neurones, delaying the onset of cell death.

Aging mammals, humans in particular, also show deregulated inflammatory responses (Bruunsgaard et al., 2001). For example, aging rats become increasingly defective at developing high fever (Plata-Salaman et al., 1998). In aging humans and patients suffering from chronic inflammatory diseases, a positive correlation exists between serum levels of Hsp70 and various inflammatory markers, such as TNF-α, C-reactive protein, and fibrinogen. This suggests that Hsp70 is directly linked to the inflammatory response, as well as to the immune and autoimmune responses (Njemini et al., 2004). For example, in rheumatoid arthritis and other spondyloarthropathies, with a pathogenesis attributed, in part, to the interaction between genetic and environmental factors, synovial cells continuously over-express Hsp70 or Hsc70 (Vargas-Alarcon et al., 2002). Hsc70 may be upregulated as a result of the high activity of these cells in several respects, including antigen processing and presentation (Schick et al., 2004). The most recent hypothesis implies a special interaction between HLA-DR chains from rheumatoid arthritis and members of the Hsp70s, which may affect antigen processing and antigen presentation (Roth et al., 2002). Indeed, the loading of the DR molecules with T-cell epitopes and presentation apparently depends on Hsc70 levels. Moreover, Hsp70 and Hsp60 have been shown to protect against cell death by interfering with the mitochondrial apoptosis pathway (Saleh et al., 2000; Zamostiano et al., 1999). Yet, when applied on the external surface of cells, Hsp70 may have a pro-inflammatory role (Asea et al., 2000; Asea et al., 2002). This is exemplified in the case of the acute respiratory distress syndrome (ARDS), which is an inflammatory response in the lungs resulting from severe damage to alveolar cells, culminating in necrosis and fatal apoptosis, especially in aging patients. Sepsis-induced ARDS in rats has been shown to correlate with the specific failure of Hsp70 to be expressed in alveolar tissues (Weiss et al., 2000). In a rat model of sepsis-induced ARDS, adenovirus-mediated transient expression of Hsp70 in the lungs, effectively prevented apoptosis and lung failure. It dramatically improved survival (Weiss et al., 2002). These effects are partly due to a mechanism whereby Hsp70 directly impairs proteasomal degradation of IkBα (Weiss Y., personal communication). Interestingly, during the viral infection that caused the Severe Acute Respiratory Syndrome (SARS) outbreak, the death toll from an ARDS was lower than 10% in patients younger than 35 yr, but reached 66% in patients older than 75 years (Ghani et al., 2005). It is tempting to speculate that the diminished ability of aging SARS patients to induce Hsp70 accumulation in septic lungs could partly account for the strong age-dependent death toll in SARS patients.

Hsp-Inducing Drugs by Partial Poisoning

Some successful attempts to reduce neurotoxic protein aggregations have been obtained with a class of chemicals that can induce a heat-shock response at physiological temperature, as a result of partial poisoning by inhibition of protein synthesis, of chaperone-mediated protein un-folding, or of the protein degradation machinery. Chinese hamster ovary cells treated with intermediate concentrations of puromycin (20 mg/mL) showed a 1.5-fold increase in the synthesis of Hsps, including Hsp70. However, higher concentrations of puromycin (e.g., 100 mg/mL) resulted in an inhibition of Hsp synthesis (Lee and Dewey, 1987). Similarly, subsaturating concentrations of specific inhibitors of Hsp90

(geldanamycin and radicicol) (Hay et al., 2004) or of the proteasome (MG-132 and lactacystin) were also shown to induce a strong heat-shock response, leading to Hsp accumulation and some anti-aggregation effects (Holmberg et al., 2000). Thus, subinhibitory amounts of geldanamycin and radicicol in mice cells increased the sodium dodecyl sulfate (SDS)-solubility of polyQ-aggregates and of α-synuclein in human and *Drosophila* cells (McLean et al., 2004). In a rat model for Parkinson's disease, exposure to the proteasome inhibitor 6-hydroxyl dopamine showed a protective effect on dopaminergic cell death. Co-treatment of lactacystin and MG-132 significantly prevented the nigral degeneration and appearance of α-synuclein and ubiquitin-positive inclusions in substantia nigra (Inden et al., 2005).

However, Hsp induction by poisoning is expectedly prone to severe adverse effects. Most inhibitors are hydrophobic and cannot be simply delivered to neural cells at precise low Hsp-inducing concentrations while being safely kept below excessive concentrations capable of inducing apoptosis. There is a significant risk that treatment with a mild excess of Hsp90 or proteasome inhibitors may induce apoptosis, as they do in anti-cancer protocols (Setsuie et al., 2005).

Anti-Inflammatory and Hsp-Inducing Drugs

As alternatives to partial poisoning drugs and problematic thermotherapies, other drugs can specifically induce a heat-shock-like response at physiological temperature, without apparent cell poisoning. Cyclopentenone prostaglandins (PGA2 and D12-PGJ2) were among the first compounds to show a strong activation of Hsp70 synthesis in HeLa cells (Ohno et al., 1988). More recently, treatment of human cells with the antiproliferative prostaglandin A1 activated the heat-shock transcription factor (Hsf1) and consequently the accumulation of Hsp70 and Hsp90 (Amici et al., 1992). Whereas a moderate inflammatory response, in part mediated by high fewer following infection (as in septic lungs) or by protein aggregation in brain tissues, may contribute to the general induction and accumulation of anti-apoptotic heat-shock proteins, an excessive deregulated inflammatory response can lead to cell death and tissue degeneration (Beere and Green, 2001; Craft et al., 2005). This suggests possible therapies for neurodegenerative diseases with anti-inflammatory drugs. Yet, because anti-inflammatory drugs may also decrease fever, they should be chosen according to their ability to maintain Hsp accumulation despite their ability to decrease fever (Marchetti and Abbracchio, 2005). This is the case for some famous commercial remedies, such as the nonsteroidal anti-inflammatory drug (NSAIDs) ibuprofen (Wang et al., 2005b), and acetyl-salicylic acid (aspirin), which can both reduce fever and inflammation while keeping a strong heat-shock like response at physiological temperatures, in terms of nuclear relocalization of Heat-shock factor-1 and of massive production molecular chaperones such as Hsp70 and Hsp90, not only in animals (Jurivich et al., 1992; Westerheide and Morimoto, 2005), but also in plants (Saidi et al., 2005). The ability of some NSAIDs to maintain high Hsp levels while decreasing unwanted effects of excessive inflammation could account for their significant healing effects on inflammation-induced neuronal loss (Marchetti and Abbracchio, 2005). For example, in HeLa cells, ibuprofen induction of Hsp70 correlates with a reduced aggregation of a polyalanine expansion mutant of poly(A)-binding protein, a hallmark of oculopharyngeal muscular dystrophy (Wang et al., 2005b). Epidemiological data have shown that constant treatment with NSAIDs reduces the risk of Parkinson's diseases by 45%, compared to patients taking NSAIDs on a nonregular basis (Schiess, 2003). Similarly, prolonged use of NSAIDs was shown to reduce the risk of developing AD and delay the onset of the disease. Studies with Flurbiprofen or ibuprofen in AD transgenic mice have shown that the effects of these NSAIDs on Aβ deposition are reached at plasma levels similar to those achieved in humans at therapeutic dosage (for a review, *see* Gasparini et al., 2004).

Other Hsp-Inducing Drugs

Recently, very old and new compounds were added to the list of least poisonous heat-shock response inducers, such as Bimoclomol (Vigh et al., 1997), Arimoclomol (Kieran et al., 2004), Arachidonic acid, Curcumin, Resveratrol (the French paradox, Delmas et al., 2005), Geranylgeranylacetone (Susuki et al., 2005) and Celastrol (Westerheide et al., 2004). Very low concentrations of these compounds can strongly induce Hsf-1 expression and heat-shock protein accumulation in eukaryotic cells, likely by way of modulating membrane fluidity or by interfering with the heat-shock signalling pathway (Jurivich et al., 1992; Vigh et al., 1997; Hargitai et al., 2003). The antiulcer drug Geranylgeranylacetone strongly induced Hsp70 in various tissues without apparent adverse effects and caused a marked inhibition of apoptosis and of oxydative damages related to ischemic heart reperfusion, renal failure and liver

transplantation (Suzuki et al., 2005). Moreover, oral uptake of Geranylgeranylacetone leads to neuroprotection against cerebral infarction in rats and alleviates polyglutamine-mediated motor neuron disease (Katsuno et al., 2005; Uchida et al., 2006). The membrane fluidizer Arimoclomol can significantly delay the progression of amyotrophic lateral sclerosis in transgenic mice overexpressing mutant human SOD-1 (Kieran et al., 2004). Micromolar amounts of Celastrol, a quinone triterpene from the Chinese pharmacopoeia, also strongly activates the heat shock transcription factor Hsf1 and chaperone accumulation in plants (Saidi and Goloubinoff, personal communication) and in human cells, with kinetics similar to heat stress (Westerheide et al., 2004). Celastrol is also effective at reversing the abnormal cellular localization of full-length mutant Huntingtin observed in striatal cells (Wang et al., 2005a).

Hsp-Inducing Peptides

Whereas external application of short insoluble peptide aggregates, such as β-amyloid, can induce neural cell death in vitro, soluble neurotrophic peptides, such as the vasoactive intestinal peptide (VIP) and the activity-dependent neurotrophic factor (ADNF), can protect neural cells from β-amyloid induced apoptosis (for a review, *see* Gozes et al., 2005). Hence, VIP-stimulated astrocytes can secrete short peptides, such as ADNF, which, already at femtomolar concentrations, can increase the levels of mitochondrial Hsp60 while protecting neurons from death associated with a broad range of toxins, including those related to Alzheimer's disease (Zamostiano et al., 1999). It is therefore possible that by way of inducing mitochondrial chaperonins, natural neuropeptides may arrest apoptosis, leading to the development of possible peptide-based drugs against protein-misfolding diseases (Gozes and Brenneman, 1996).

Conclusions

Molecular chaperones are fold-controlling factors (*see* Table 1) that can specifically recognize misfolded or alternatively folded protein structures in the cell. Chaperones can single out and remove atypical, potentially toxic, and infectious protein structures by various cellular programs:

1. *Prevention:* binding chaperones can bind misfolded proteins and prevent them from forming large aggregates.
2. *Passive unfolding:* aggregate-interfering compounds can destabilize aggregated proteins.
3. *Passive protection:* binding chaperones can bind hydrophobic surfaces of already formed aggregates and protect membranes and proteins from toxic interactions.
4. *Active unfolding:* ATPase chaperones can forcefully unfold misfolded and aggregated proteins and allow their native refolding into nontoxic functional proteins (*see* Table 1).
5. *Sequestration:* the aggresome can actively sequester by compaction toxic misfolded species into inclusion bodies.
6. *Secretion:* toxic aggregates such as Aβ–amyloids or PrP^{sc} can be secreted outside the cell.
7. *Controlled degradation*: chaperone-gated proteases degrade and recycle non-recoverable damaged proteins (*see* Fig 1).

In aging cells, or during abiotic stress or pathogen attack, the chaperone and protease network may become overloaded because of massive protein damage, deficient responsiveness, and insufficient Hsp accumulation, or because of mutations in substrate proteins or in the chaperones (Fig 2). Several therapeutic approaches were discussed here that can potentially improve neurone responsiveness to toxic protein aggregation or to heat- and oxidative stresses, thereby increasing the cellular levels of Hsps. Combinations of treatments such as caloric restriction, mild heat-shocks, peptides, and drugs capable of increasing chaperone and protease levels without overloading or poisoning the cell, and without increasing the amount of protein aggregates in the cell, are likely to improve cell survival to protein misfolding and reduce neuronal loss. In addition to treating various neurodegenerative diseases, Hsp-inducing treatments, possibly in combination with dietary antioxidants (for a review, *see* Cui et al., 2004), may also relieve other protein misfolding and inflammatory diseases such as cystic fibrosis (Gelman and Kopito, 2002), diabetes (Hayden et al., 2005), and, in general, aging (for a review, *see* Macario and Conway de Macario, 2005).

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