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REVIEW

Protein Folding, Misfolding, and Aggregation. Formation of Inclusion Bodies and Aggresomes

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Abstract—In this review the mechanisms of protein folding, misfolding, and aggregation as well as the mechanisms of cell defense against toxic protein aggregates are considered. Misfolded and aggregated proteins in cells are exposed to chaperone-mediated refolding and are degraded by proteasomes if refolding is impossible. Proteolysis-stable protein aggregates accumulate, forming inclusion bodies. In eucaryotic cells, protein aggregates form structures in the pericentrosomal area that have been termed "aggresomes". Formation of aggresomes in cells is a general cellular response to the presence of misfolded proteins when the degrading capacity of the cells is exceeded. The role of aggresomes in disturbance of the proteasomal system operation and in cellular death, particularly in the so-called "protein conformational diseases", is discussed.

Key words: folding, misfolding, aggregation, inclusion bodies, aggresomes, chaperones, proteasomes, microtubules

Protein aggregation *in vitro* and *in vivo* arises as a result of protein misfolding in response to mutations, posttranslational modifications, or changes in local conditions such as pH and temperature. Because protein misfolding and aggregation into insoluble intracellular complexes and inclusion bodies are problems that must be considered in the solution of biotechnological tasks and because they underlie pathogenesis of a considerable part of human degenerative and neurodegenerative diseases [1-4], special attention has been given in recent years to investigation of protein misfolding and aggregation. Such disorders as Alzheimer's and Parkinson's diseases [5, 6], Huntington's disease [7], Machado–Joseph's disease (spinocerebellar ataxia) [8], prion encephalopathies [9, 10], Charcot's disease (amyotrophic lateral sclerosis) [11, 12], systemic amyloidosis [13], and cystic fibrosis [14] are part of a growing list of inherited diseases known as "conformational diseases" [5] that arise when proteins undergo structural rearrangement promoting their aggregation. A distinctive feature of these diseases is alteration of protein conformation that results in the formation of protein aggregates with various supramolecular organization [15]. Formation of protein aggregates in cells proceeds by different molecular mechanisms, and understanding of these processes opens up new prospects for investigation and treatment of "conformational diseases".

The goal of the present review is to summarize literature data of recent years on mechanisms of protein folding, misfolding, and aggregation as well as on mechanisms of cellular response to formation of non-native protein aggregates.

PROTEIN FOLDING AND MISFOLDING

Native, functionally active proteins form by folding of newly synthesized polypeptide chains, which results in information transfer from the linear chains of amino acids to a three-dimensional structure [16]. Protein folding is defined by kinetic as well as thermodynamic factors [17, 18]. Kinetic features of folding are associated with the vectorial nature of protein synthesis, and the thermodynamic features arise due to the necessity of energy minimization of folded polypeptide chain [19, 20]. The native structure of polypeptide, resulting from folding, is the thermodynamically most stable conformation [21-24].

Protein folding and misfolding *in vitro*. Native proteins can unfold under the effect of denaturing agents such as guanidine hydrochloride or urea as well as due to thermal denaturation. Many denatured proteins fold

Abbreviations: MTOC) microtubule-organizing center; SOD) superoxide dismutase; UPS) ubiquitin–proteasome system; ER) endoplasmic reticulum.

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spontaneously, and refolding efficiency may be controlled by dilution of protein solution and by decrease of the folded protein concentration [25]. Small single-domain proteins, in which hydrophobic amino acid residues are buried in the protein molecule, fold through one-stage process in less than 1 sec without formation of intermediates [17, 26-30]. Refolding of multi-domain proteins usually proceeds with formation of one or more intermediates [31-33].

Different models have been established to explain the extremely high speed of this process [34, 35].

1. According to the framework model [36, 37], protein folding starts with formation of elements of secondary structure independently of tertiary structure or, at least, before completion of the formation of the latter. Elements of secondary structure then assemble into the tightly packed native tertiary structure either by diffusion and collision [30, 38] or by propagation of the structure [39].

2. According to the hydrophobic collapse model [40-42], the initial stage of the folding is a relatively uniform collapse of the protein molecule, mainly driven by the hydrophobic effect. Stable secondary structure starts to grow only in the process of collapse.

3. According to the nucleation–condensation mechanism [43-47], a previously formed diffuse nucleus of folding polypeptide chain catalyses further folding. The nucleus primarily consists of several adjacent residues, which form an almost correct secondary structure. However, such nucleus is stable only on interactions realized during formation of correct tertiary structure. Consequently, interactions inherent to secondary and tertiary structures determine the folding transition state, which is structurally similar to the native state, being formed as a result of collapse (condensation) around a diffuse nucleus. As the propensity of folded polypeptide chain for stable secondary structure increases, folding becomes more hierarchical and eventually proceeds by a framework mechanism where the transition form is assembled from pre-formed secondary structural elements.

Inasmuch as formation of native state by numerous multi-domain [48] and by some single-domain proteins [19] is linked with overcoming of a kinetic barrier, folded proteins become "kinetically trapped" in local energetic minima [49]. In this connection, folding of multi-domain proteins may be accompanied by production of incompletely folded or misfolded intermediates [50]. Hydrophobic amino acid residues and segments of unstructured polypeptide backbone are frequently exposed to the solvent. As a consequence, hydrophobic interactions and hydrogen bonds between non-native polypeptide chains can lead to formation of disordered complexes [29, 51].

Protein folding and misfolding *in vivo*. *In vivo*, protein folding differs significantly from *in vitro* folding. This difference is due to quite dissimilar *in vivo* and *in vitro* conditions during the folding of a polypeptide chain. At the time of *in vivo* protein folding, molecular chaperones, abundant in all cellular compartments where folding of newly synthesized nascent chains proceeds, minimize the contribution of off-pathway reactions that result in formation of protein aggregates [52-54]. Molecular chaperones in cells participate in folding of newly synthesized nascent chains, as well as in assembly and decomposition of supramolecular protein structures [32, 52, 55-60]. Many molecular chaperones, known as heat shock proteins (Hsp), are synthesized in cells in response to stress (heat, oxidative, or toxic) and comprise several classes of proteins of conserved amino acid sequence—Hsp100 (ClpA/B/X, HslU), Hsp90 (HtpG), Hsp70 (DnaK), Hsp60 (GroEL), and small Hsp's (shown in parentheses are *Escherichia coli* chaperones of corresponding classes) [25, 58, 61]. Such chaperones as Hsp70 and Hsp60 interact transiently with unfolded or partially folded intermediates, prevent their hydrophobic surfaces from "incorrect" intermolecular and intramolecular interactions, and favor folding of polypeptide chains [25, 58, 60]. There are data showing that the DnaK chaperone system (DnaK, DnaJ, GrpE) can also participate in ribosome assembly in *E. coli* cells and can increase the rate of that process [62]. Some ribosome components, 23S-RNA in particular, function as molecular chaperones, promoting folding of newly synthesized chains [63, 64].

In vivo two folding mechanisms, cotranslational and posttranslational, are possible. According to the cotranslational folding concept, folding of newly synthesized chains in cells proceeds during ribosomal synthesis [63-67]. Folding of nascent chains can proceed in the ribosome tunnel, where polypeptides are protected from aggregation and degradation. Binding of nascent chains with chaperones, including trigger factor, Hsp70, and prefoldin, stabilize elongating chains on ribosomes in a non-aggregated state [50]. Newly synthesized chain can complete folding into biologically active conformations in cytosol after the protein emerges from the ribosome. The cotranslational folding mechanism of newly synthesized chains is characteristic of both procaryotic and eucaryotic cells, and appears to be the universal and the most evolutionarily ancient mechanism [65, 66]. According to the posttranslational folding concept, nascent chain begins to fold after complete biosynthesis and release from the ribosome [68-70]. Speaking for the protein posttranslational folding concept are the results of *in vitro* renaturation of small globular denatured proteins, which quickly regain their native structure after removing a denaturing agent, as well as the possibility of obtaining native protein analogs by chemical synthesis [71].

The *in vivo* protein folding mechanism has not been studied sufficiently. The fate of polypeptides as to whether they fold correctly into native functionally active molecules or form aggregates is determined by competition

between protein folding, degradation, and aggregation [72]. The yield of *in vivo* folded proteins is low, possibly due to transformation of folding intermediates into misfolded intermediates, which are prone to formation of insoluble aggregates [73]. Concentrations of macromolecules inside the cell (macromolecular crowding), as high as ~340 g/liter, can also facilitate the aggregation of non-native polypeptide chains mainly due to their real concentration increase [31, 55, 74]. Crowding may lead to compacting and association of folded macromolecules [74], including collapse of polypeptide chains [75] and interaction of non-native polypeptides with chaperones [50]. Variation of environmental conditions (pH, temperature, ionic strength, redox state) as well as inhibition of the protein degradation system can lead to formation of misfolded intermediates [76, 77].

PROTEIN AGGREGATION

Protein aggregation *in vitro* and *in vivo*. It has been accepted that protein aggregation occurs on "incorrect" interactions of completely or partially unfolded protein molecules, leading to formation of agglomerates of random shape [78]. Aggregates are produced when folded or unfolded intermediates interact mainly through contact of their hydrophobic surfaces, turning into large, stable complexes [23, 79-81]. Structured or amorphous aggregates produced [73] are defined by poor solubility in water or detergents and by non-native secondary structure [1]. Aggregated proteins are functionally inactive and in many cases are rich in antiparallel β-chains [82].

The mechanism of protein aggregation together with unfolding of a protein molecule involves the stage of the aggregate seed formation (nucleation stage), and the stage of aggregate growth [83, 84]. If one supposes the indestructibility of growth points of the aggregates, one might draw an analogy between the process of protein aggregation and chemical chain reactions, such as oxidation by molecular oxygen, chlorination and bromination of many compounds, reactions of the thermal decomposition, and many reactions of chain polymerization [84, 85]. Free radicals, participating in the chain reaction, are called active centers of that reaction. Active centers of the chain reaction at protein aggregation are the growth points of the aggregate. It follows from comparison of the protein aggregation process with chemical chain reactions that aggregation (at the stage of aggregate growth) may be considered as an unramified chain reaction, and the chaperones, blocking the aggregation of the protein substrate, as inhibitors of the chain reaction of protein aggregation [84]. Based on investigation of the tobacco mosaic virus coat protein and firefly luciferase, new concepts of aggregation kinetics have been formulated [84-86]. Concepts about two kinetic regimes of protein aggregation have been introduced. At rather low protein concentrations, a kinetic regime is realized, when the rate-limiting stage is the stage of aggregate growth on nuclei formed in the nucleation stage. At rather high protein concentrations, a kinetic regime is realized when the rate-limiting stage is the stage of complete or partial protein molecule unfolding.

In vivo protein aggregation proceeds both in the intracellular compartments and in the extracellular space when formation of misfolded proteins exceeds the cellular capacity to degrade them [87]. In cells, protein aggregation can arise from misfolding resulting from amino acid misincorporation into polypeptides due to mutations or errors in transcription, mRNA processing, or translation [13, 88]. Besides, environmental factors such as thermal stress [89], osmotic and oxidative stress [90], as well as products of viral genes [91, 92] can interfere with the folding of nascent polypeptides and cause protein aggregation. Finally, unequal synthesis of subunits of heterooligomeric protein complexes can lead to the production of unassembled polypeptide chains [93]. Many misfolded and unassembled proteins in cells expose hydrophobic areas on their surface, which, in native proteins, are buried deep in the molecule, and, as a result, protein regions that can interact leading to aggregate formation increase in number [80]. Failure in protein translation and in protein integration into membranes can result in exposure of extensive protein side chain hydrophobic stretches, normally embedded in the lipid bilayer, to the cytosol aqueous environment. Inappropriate exposure of hydrophobic sequences leads to formation of non-native conformations that can interact with existing aggregates [13]. The aggregates can be either amorphous structures, such as inclusion bodies, or ordered fibrils such as amyloid plaques and prion particles (Fig. 1) [73, 94-96]. Amyloid fibrils formed from various proteins have common structure independent from protein amino acid sequences and consist of small protofilaments formed by polypeptide chains stacked in β-folded sheets, which run perpendicular to the longitudinal axis of the fibril.

Protein homo- and hetero-aggregates. There is evidence showing that in a system containing more than one protein, aggregation takes place only among identical or very similar polypeptides, and cohesive aggregates may form due to the specific domain-swapping interaction [97, 98]. In this case, dissimilar co-aggregating polypeptides do not interact with each other and rather inhibit rather than accelerate each other's aggregations [99]. Indeed, it has been shown that P22 tail spikes and coat proteins do not form hetero-aggregates [100]. It follows from refolding studies of chemically denatured polypeptides that protein aggregation *in vitro* is due to specific intermolecular interactions among defined domains within structured intermediates [101-103].

Kopito and coauthors [104] studied an ability of unrelated misfolded proteins, specifically, hydrophobic

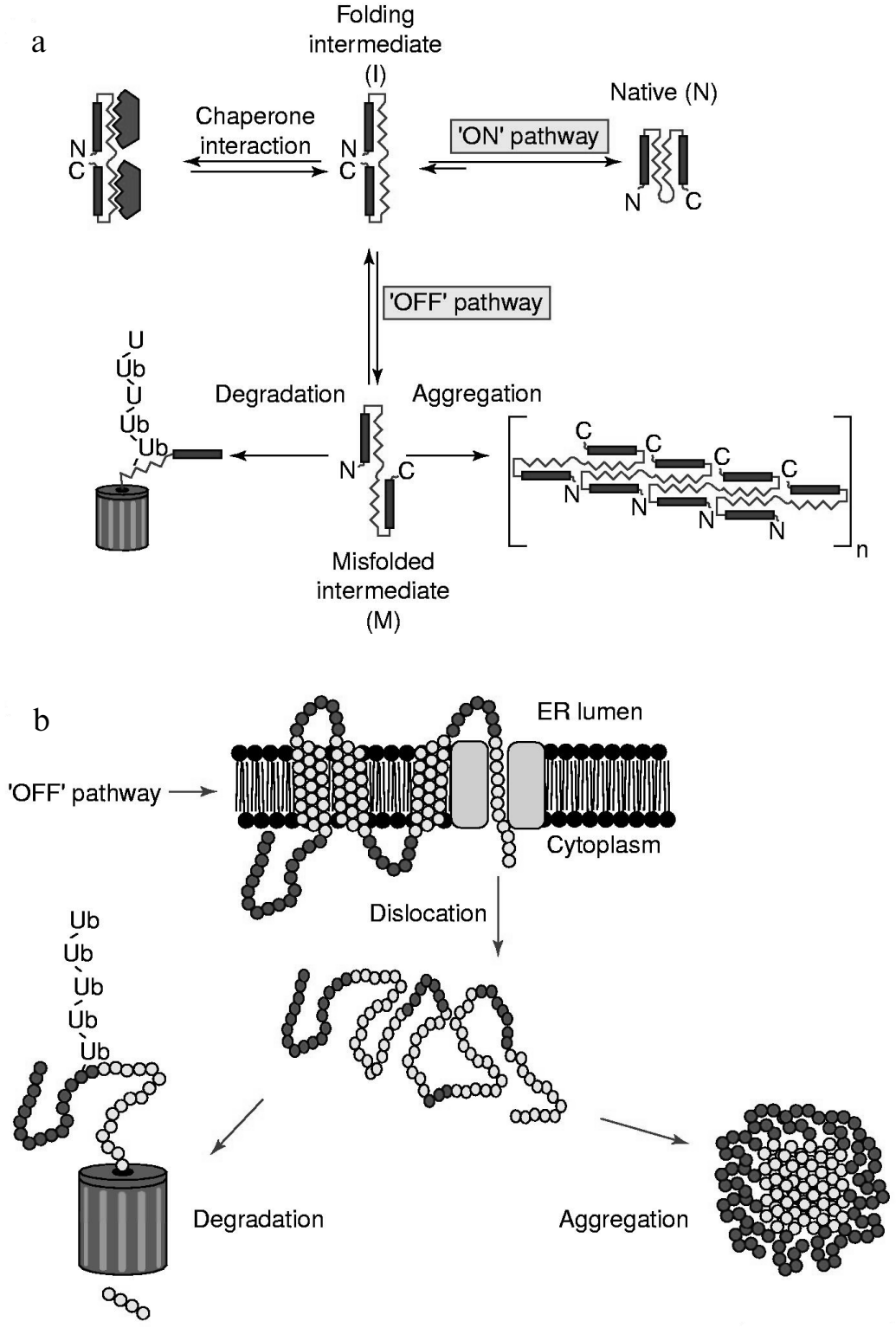

Fig. 1. *In vivo* formation of protein aggregates from kinetically trapped products of protein folding or degradation [73]. a) Formation of structured (amyloid) aggregates. Folding intermediates can assume misfolded off-pathway conformations that are prone to form aggregates. b) Formation of amorphous aggregates. Misfolded integral membrane and secretory proteins are dislocated across the endoplasmic reticulum (ER) membrane to the cytoplasm for degradation by the ubiquitin–proteasome pathway, or produce amorphous aggregates.

proteins (the F508 mutant of the cystic fibrosis transmembrane conductance regulator (CFTR), α-chain of T-cell receptor) and hydrophilic protein huntingtin expressed in human embryonic kidney cells 293 (HEK293) to co-aggregate with the hydrophobic protein P23H rhodopsin. Two independent approaches have been used for this purpose. One approach, based on the deconvolution microscopy method allowed to assess the intracellular localization of aggregated proteins expressed in the same cell. The second approach, based on fluorescence resonance energy transfer (FRET), has involved the use of expressed aggregation-prone hybrid proteins produced due to fusion with fluorophore proteins. This method allowed assessing the degree of co-aggregation of hydrophobic proteins with hydrophilic proteins. As a result of these investigations it has been determined that, firstly, protein aggregation in cells occurs in separate centers specific for each protein type, and, secondly, aggregation exhibits specificity even among hydrophobic proteins expressed at high levels. Thus, in cells, aggregation of misfolded proteins is rather a result of specific self-association of similar proteins than nonspecific interaction among unrelated proteins. It is suggested that specificity of hydrophobic protein aggregation reflects specificity of aggregation among partially folded (misfolded) intermediates. However, because rhodopsin and mutant form of CFTR are integral membrane proteins, whose hydrophobic domains are not exposed to cytosol, it is likely that intermediates leading to aggregation are different from those that are produced according to the native folding pathway.

At the same time, data are available about heterologous protein aggregation. Thus, Steffan et al. [105] observed heterologous aggregation between huntingtin and rhodopsin in the mammalian cells. According to the results of Goloubinoff and coauthors [106], cross-interactions between dissimilar proteins can take part in a protein mixture, and one protein is able to affect the other's kinetics of aggregation. On the example of slow-aggregating glucose-6-phosphate dehydrogenase and fast-aggregating malate dehydrogenase, it has been demonstrated that aggregates formed due to thermal denaturation of one fast-aggregating protein can associate with another slow-aggregating protein and accelerate that aggregation. Such proteinaceous infectious behavior is similar to that of yeast prions, when one prion protein affects the aggregation of other prions [107, 108].

Chaperone-mediated disaggregation of protein aggregates. Protein aggregates that are formed and accumulated in cells due to continuous action of unfavorable factors, such as thermal stress, can be disaggregated and resolubilized in the presence of a number of specific ATP-hydrolyzing chaperones and co-chaperones: Hsp100 (ClpB) and Hsp70/Hsp40/Bag1 (DnaK, DnaJ, GrpE) [109, 110]. Participation of molecular chaperones in detoxication of protein aggregates has been established

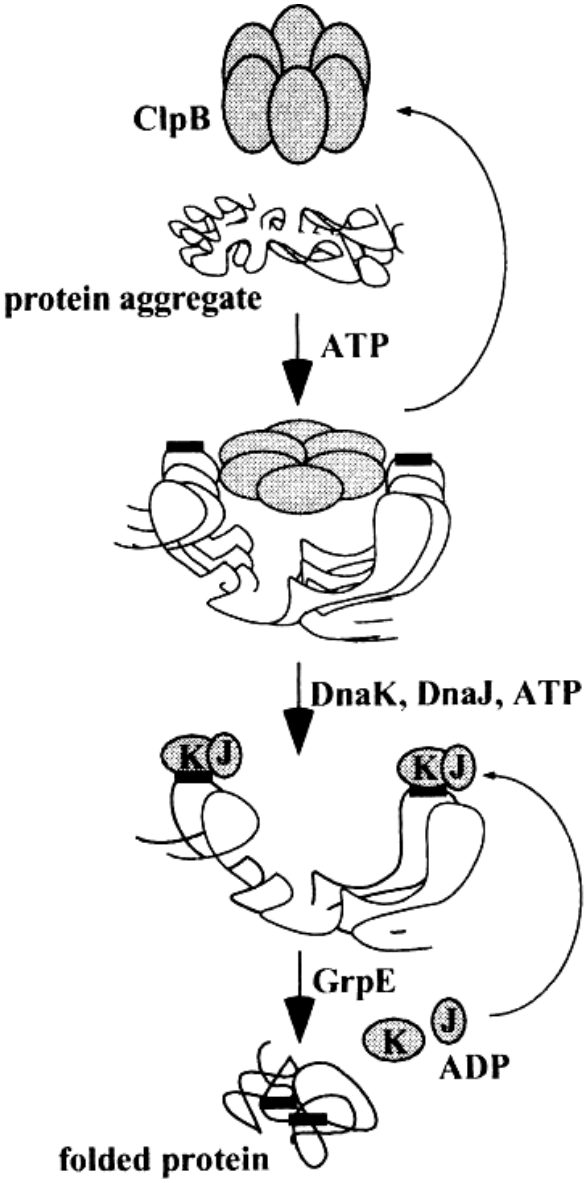

Fig. 2. Mechanism of disaggregation of protein aggregates by DnaK-DnaJ-GrpE [109] (see text).

both for procaryotic and eucaryotic members [109, 111-113]. Thus, for example, overexpression of Hsp100 and/or Hsp70 chaperones leads to solubilization of polyglutamine-rich protein aggregates formed under stress in yeast and in animals [114]. In *E. coli* cells, degradation and refolding of insoluble aggregates proceed in several stages assisted by the DnaK chaperone and by DnaJ, GrpE, and ClpB co-chaperones, as well as the GroEL chaperone and the GroES co-chaperone [112, 115], by a mechanism comprising sequential action of these chaperones (Fig. 2) [109, 112, 116]. The first stage of this process includes melting of surfaces of large compact aggregates by ClpB, accompanied by exposure of new DnaK-binding sites on the surfaces of aggregates,

with which DnaK interacts. As a result of DnaK interaction with these sites, hydrophobic associations between aggregated polypeptides weaken and that leads to their unfolding. ATP hydrolysis and DnaK release are further accompanied by local intramolecular refolding of native domains, leading to a gradual weakening of improper intermolecular links. Therewith, as shown in *in vitro* experiments, the effectiveness of the chaperone-mediated disaggregating of protein aggregates depends on protein nature, aggregate size, and its solubility [110, 112, 115].

Villaverde and coauthors [117] studied the involvement of individual chaperones in protein aggregate and inclusion body formation by producing the misfolding-prone hybrid VP1LAC protein (a derivative of β-galactosidase) in chaperone null mutant cells of *E. coli*. Unexpectedly, it was found that the absence of the GroEL chaperone significantly reduced the level of aggregated protein VP1LAC and favored the incidence of the soluble protein form, from 4 to 35% of the total VP1LAC protein. However, instead of regular inclusion bodies, abundant small aggregates were formed. On a background of DnaK in cells, the amount of aggregated protein was 2.5-fold higher than in the wild-type cells. It follows from these data that GroEL and DnaK chaperones appear as major antagonist regulators of inclusion body formation. DnaK prevents inclusion body formation by reducing the aggregation degree of misfolded protein, whereas GroEL drives the protein transit between the soluble and the insoluble cell fractions and participates in inclusion bodies formation. It is suggested that an analogous process takes place in eucaryotic cells *in vivo* under the effect of homologous chaperone family Hsp60 in cases of conformational diseases [118, 119].

Thus, chaperone-dependent disaggregation of protein aggregates can serve as a central cellular tool for the recovery of native proteins from toxic aggregates, such as polyglutamine-rich proteins, amyloid plaques, and prions [113]. A cascade of intracellular modifications leading to protein aggregation is observed at low expression of Hsp type chaperones, for example in apoptosis [120] or aging [121].

INCLUSION BODIES AND AGGRESOMES

Inclusion bodies. In cells, aggregated proteins can accumulate as discrete depositions termed inclusion bodies [13]. Recombinant proteins, obtained biotechnologically, also aggregate frequently and form inclusion bodies [73, 122, 123]. Inclusion bodies consist of compact packed protein molecules, partially possessing secondary structure [124]. In today's view, inclusion body formation in procaryotic and eucaryotic cells proceeds by different mechanisms. In procaryotic cells, aggregation is seeded at a single or limited number of sites, and inclusion bodies form due to assembly of non-native monomers into growing polymers [125]. Because the addition of monomers to existing aggregate favors the thermodynamic stability of the aggregate, initially arisen aggregates will tend to dominate. In this connection, the number of aggregation foci or inclusion bodies in the cell will be limited. According to this model, inclusion bodies in procaryotic cells are giant aggregates, formed by monomer diffusion to the site of inclusion body formation. Inclusion body formation was formerly considered to occur passively by the irretrievable deposition of partially folded intermediates. However, according to the data of Villaverde and coauthors [126-128], aggregation of recombinant proteins in bacteria occurs as a reversible process deeply integrated in the cell mechanisms for coping with thermal stress. Inclusion body formation might actually be supported by the cellular machinery that operates under specific stress conditions, promoting transient accumulation of misfolded polypeptides until they could be further processed, either refolded or proteolyzed.

In eucaryotic cells, an inclusion body is a structured complex of aggregates [73]. Such inclusion bodies arise from the coalescence of individual aggregates into a single or limited number of foci. Cytoplasmic inclusion bodies in animal cells often result from the expression of mutant or heterologous proteins, and in the course of a number of various diseases.

Protein quality control. Protein aggregates do not accumulate under normal, physiological conditions, despite their continued production in cells, due in part to the existence of cellular "quality control" machinery, which involves a joint effect of chaperones and proteinases [129-131]. Protein quality control refers to the "control system" of protein folding and protein assembly in the endoplasmic reticulum (ER), which ensures correct folding of proteins and their following maturation in final compartments of the secretory pathway [132]. Nascent proteins that fail to fold or assemble in the ER are degraded by a process known as ER-associated degradation (ERAD) [130, 133, 134]. Studies on mammalian cells and on yeast have led to the formulation of a mechanism for ERAD, in which protein substrates are degraded by proteasomes following retrotranslocation (i.e., "dislocation") across the ER membrane by a process in which the Sec61 translocon and functional ubiquitination machinery at the cytosolic face of the ER membrane participate [135-137]. Dislocation of ERAD substrates across the ER membrane is kinetically coupled to their degradation by proteasomes, and, in many cases, cytosolic intermediates of degradation are not detected [138]. Thus, membrane and secretory proteins that fail to fold or assemble properly are dislocated from the ER to the cytoplasm, where they are subjected to degradation by proteasomes [139, 140]. It is suggested that ubiquitin-proteasome-mediated degradation of incorrectly folded proteins in mammalian cells is the dominant ER-associated pathway for disposal of misfolded proteins [140]. This mechanism works to

degrade both inefficiently folded mutant proteins, such as F508 mutant of cystic fibrosis transmembrane conductance regulator (CFTR) [140, 141], and misfolded [142-144] or incorrectly assembled [145] wild-type proteins. Under conditions of disturbed proteolysis, proteasomes and ubiquitinated proteins are accumulated and form aggregates or inclusion bodies.

The proteolysis centers and formation of aggresomes. Up to 30% of the intracellular ribosome-synthesized proteins are defective and are degraded shortly after their synthesis [146]. Most defective proteins in mammalian cells are degraded by the ubiquitin–proteasome system (UPS), especially by 26S proteasomes, usually after protein tagging with a polyubiquitin chain [131, 147]. In eukaryotic cells, proteasomes are present throughout the cytoplasm and also found in the nucleus [148-150]. Cytoplasmic proteasomes are associated with the centrosomes, cytoskeletal networks, and the outer surface of the ER [14, 77, 151]. Some of the intracellular compartments are characterized by increased proteasome-dependent proteolytic activity and are therefore termed "proteolysis centers" [150, 151]. The hypothesis about the existence in cells of proteolysis centers was proposed after discovering that treatment of different cell lines with a selective proteasome inhibitor caused the formation of a single perinuclear aggregate, which contained proteasomes and ubiquitinated proteins, rather than a generalized accumulation of ubiquitinated proteins throughout the cell [151].

The formation of large perinuclear inclusion bodies containing protein aggregates was first described in HeLa cells [151]. Wojcik et al. [151] found that treatment of HeLa cells with a proteasome inhibitor (N-benzyloxycarbonal-Ile-Glu(O-*t*-butyl)-Ala-leucinal) results in the accumulation of electron-dense structures in the vicinity of the Golgi apparatus. Recently, perinuclear inclusion bodies very similar to the ones described by Wojcik et al. in HeLa cells [151] have been discovered in other mammalian cell-culture systems [14, 77, 152-154].

It has been shown that formation of cytoplasmic inclusion bodies in mammalian cells is associated with retrograde transport of misfolded protein on microtubules, which participate in the growth of protein aggregates within the cytoplasm (Fig. 3) [14]. Kopito and coauthors [14] proposed calling microtubule-dependent cytoplasmic inclusion bodies "aggresomes". Aggresomes are pericentrosomal structures of 60-80 nm, containing misfolded, aggregated, ubiquitinated proteins [14, 73, 155]. Depolymerization of the microtubules with nocodazole prevents formation of the perinuclear aggresomes and induces the production of small protein aggregates that are dispersed throughout the cytoplasm [14].

The formation of aggresomes can be induced not only by the inhibition of proteasomes, but also by overexpression of proteins normally degraded by proteasomes. Attempts to express integral membrane proteins in mammalian cell culture frequently result in mistargeting of heterologous proteins to cytoplasmic aggresomes. In particular, it has been demonstrated that heterologous expression of CFTR leads to the formation of SDS-insoluble aggregates, which accumulate in a single juxtanuclear aggresome, co-localized with centrosome markers [14, 155]. Electron microscopy has shown that CFTR-containing aggresomes consist of electron-dense, membrane-free particles and are localized in an area adjacent to the centriole [73].

Dynamics of aggresome formation. Formation of aggresomes in COS-7 cells expressing a cytosolic protein chimera GFP-250 (green fluorescent protein), which was fused at its C-terminus to a 250-amino acid fragment of membrane transporter p115, has been observed by equipment that provides optical time-lapse images of particles in living cells [77]. As a rule, small protein aggregates originating within the periphery of the cell travel to the MTOC region where aggresomes are formed. Aggregate particles move at speeds exceeding those of simple diffusion [77]. Estimation of the rate of aggresome growth based on fluorescence intensity of the juxtanuclear aggresomal compartment demonstrated that fluorescence intensity after 2 h is doubled in comparison with the initial intensity. It follows from these data, that the fluorescence intensity of the aggresome increases linearly over time, and aggresome formation follows first order linear kinetics [77].

Motility of peripheral aggregates is defined by the state of microtubules, and after treatment of cells with nocodazole, disrupting microtubules, aggregates lose their motility [14, 77]. Transport of ATP-hydrolysis-driven organelles and protein particles along cytoskeletal filaments in eukaryotic cells is performed by molecular motors (kinesin, myosin, and dynein) [156]. Minus-end-directed transport of protein particles along microtubules requires dynein, which is typically associated with dynactin. Dynein/dynactin-associated minus-end motor activity of microtubules can be inhibited by over-expressing the p50/dynamitin subunit of the dynactin complex, causing dissociation of the dynein/dynactin complex [157, 158]. To test if the dynactin complex is involved in the transport of peripheral aggregates to the MTOC, COS-7 cells were co-transfected with GFP-250 and p50/dynamitin, and the aggresome formation was examined in cells expressing both proteins [77]. Aggresome formation was found in 58% of cells transfected only with GFP-250, but 18% of cells co-transfected with GFP-250 and p50/dynamitin and expressing high levels of p50/dynamitin, showed aggresome formation. In most of the co-transfected cells, GFP-250 aggregates were detected in the periphery of the cell. Thus, overexpression of p50/dynamitin of the dynein/dynactin complex prevents formation of aggresomes even in the presence of the proteasome inhibitor [77, 159]. It follows from the latest data that dynein-dependent retrograde transport of peripheral aggregates to the MTOC plays a key role in aggresome

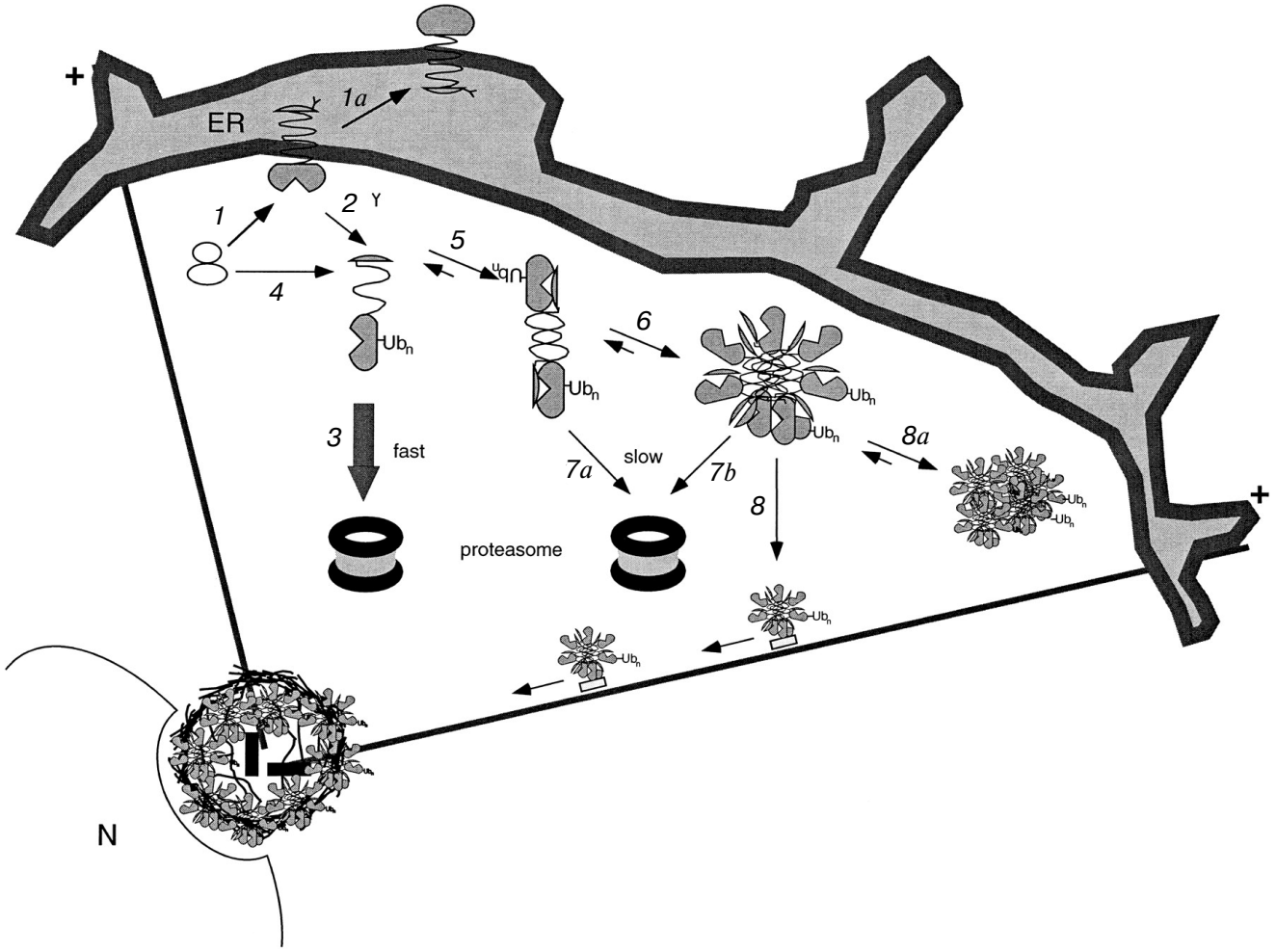

Fig. 3. Model for aggresome formation [14]. Numbers indicate various steps in the aggresome biogenesis pathway. Integral membrane proteins are cotranslationally translocated to the membrane of the ER (*1*). Some molecules fold to adopt a maturation-competent conformation (*1a*). Others misfold, are dislocated from the ER membrane, and deglycosylated by cytoplasmic N-glycanase (*2*). Some proteins may escape the translocation machinery and be delivered directly to the cytoplasm (*4*). Dislocated from membrane of ER, ubiquitinated misfolded proteins can either be degraded by cytosolic proteasomes (*3*) or aggregate (*5* and *6*). Because aggregates are difficult to unfold, they are slowly degraded by proteasomes (*7a* and *7b*). Misfolded aggregated proteins are transported to the microtubule-organizing center (MTOC) by microtubules where they become entangled with collapsed intermediate filaments (*8*). In the absence of microtubules, protein aggregates coalesce at dispersed sites throughout the cytoplasm (*8a*). Accepted designations: ER, endoplasmic reticulum; N, nucleus; Ub_n, ubiquitin conjugates; symbol + designates (+)-end of microtubules.

formation [77, 159]. Video time-lapse microscopy [77] and immunogold electron microscopy [159] data evidence that aggresomes form as a result of retrograde transport of aggregated proteins along microtubules.

Composition of inclusion bodies and aggresomes. Bacterial inclusion bodies typically contain a single species of aggregated protein [106]. However, as histochemical studies have demonstrated, aggresomes are complex structures that contain subcellular organelles and filaments, various proteins, including molecular chaperones (Hsc70 and Hsp40), the chaperonin TriC/TCP [77, 155], centrosome material, proteins of cytoskeleton as well as UPS components (especially, 19S and 26S proteasome subunits), and are surrounded by a network of intermediate filament protein vimentin [73, 155]. The organelles within aggresomes appear to be predominantly mitochondria and lysosomes. The most consistent components of aggresomes are intermediate filament proteins that in cells containing aggresomes diverge from their normal cellular distribution. Thus, for example, vimentin (component of type-III intermediate filaments) forms a cage-like structure wrapped around the aggresome [14]. The role of this vimentin cage for aggresome formation is unknown, however it is suggested that this structure might contribute to the stability of aggresomes [73]. Aggresomes might also be enriched in proteins of the signal system and proteins participating in cell division and in apoptosis. Complexity of composition of

inclusion bodies in mammalian cell cytoplasm is either a result of protein co-aggregation or reflects the complexity of the mechanism of inclusion body formation.

Effect of aggresomes on cellular structure and function. Aggresomes are usually localized in the cell region that also contains the Golgi complex, and might interfere with correct Golgi localization [77]. Golgi complex becomes more disordered as the aggresome grows, and instead of the normally compact Golgi structure surrounding the MTOC, the Golgi elements disperse around the aggresome. It is suggested that aggresomes form a barrier between Golgi cisternae and MTOC, around which they are usually aligned [77]. Similarly, the microtubular network is partially disorganized, with microtubules surrounding the aggresome instead of originating from the MTOC [77]. However, despite these changes in the aggresome containing cells, many intracellular processes do not deviate from the norm. All processes of protein synthesis and quality control, protein transport from the ER to the Golgi complex, observed within normal cells, occur normally in aggresome-containing cells [77].

PROTEIN AGGREGATES, INCLUSION BODIES, AGGRESOMES, AND NEURODEGENERATIVE DISEASES

In neurons, deposition of misfolded proteins with inclusion bodies formation is a common feature underlying many neurodegenerative diseases, suggesting that these diseases may share common mechanisms relating to the failure of neurons to eliminate misfolded proteins [160]. Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, prion encephalopathies, amyotrophic lateral sclerosis, cystic fibrosis, and many others are associated with aggregation of polypeptide chains of misfolded proteins, forming insoluble nuclear and cytoplasmic inclusion bodies which are toxic to cells [95, 161-166]. In a pathologic process, in some cases, more than one protein coexisting in inclusion bodies is involved. Often, proteolytically degraded fragments are more prone to forming aggregates than native precursor proteins.

The autopsy of patients' brains with Alzheimer's disease revealed the presence of two types of lesions: extracellular deposits forming neuritic plaques, which are deposits of β-amyloid peptide (βA) [167], and intracellular bundles of neurofibrillary tangles, which are clusters composed of paired helical hyperphosphorylated filaments of tau protein associated with the neuronal microtubules [168-170]. βA with molecular mass of 4.2 kD is a degradation product of the transmembrane amyloid precursor protein (APP). Processing of APP proceeds by α-, β-, and γ-secretases [171]. As a result of APP processing under normal, physiological conditions soluble form of βA (1-40) is produced which circulates extracellularly and does not aggregate [165]. In the inheritable (familial) form of Alzheimer's disease, which is linked to mutation in the APP encoding gene and to mutations in genes of both presenilins 1 and 2, hydrophobic βA (1-42) is produced [172]. βA (1-42) readily aggregates and seeds the formation of amyloid fibrils that can act as templates for formation of amyloid plaques [173]. βA-fibrils in mature plaques are disposed in the plaque core and are surrounded with fragments of nerve filaments and with activated microglia [170]. Buckig et al. [87] have demonstrated on cell culture expressing βA_{42} in ER, that a βA_{42} fraction can be exported from the ER to the cytosol, where it forms large aggregates containing ubiquitin, proteasomes, and Hsc70 chaperone, and these aggregates share several constituents with aggresomes. However, aggregates formed by βA_{42} are distinct in their cellular localization from aggresomes in that they do not accumulate around the centrosome but are distributed randomly around the nucleus.

Parkinson's disease is characterized by formation of cytoplasmic protein inclusion bodies known as Lewy bodies and by loss of midbrain dopaminergic neurons [174, 175]. The development of Parkinson's disease is associated with deposition of both α-synuclein [176, 177] and parkin proteins [175], which are found in Lewy bodies. It is suggested that formation of α-synuclein and parkin deposits is associated with mutations of these proteins and with their disturbed ubiquitination [178]. Overexpression of parkin in the presence of a proteasome inhibitor leads to formation of aggresome-like perinuclear inclusions [175] with distinctive intrinsic structure and with immunoreactivity to α-synuclein, parkin, ubiquitin, molecular chaperones, and 26S-proteasome subunit [175]. Besides, data are available showing that Lewy bodies contain aggresome-related proteins γ-tubulin and pericentrin, the ubiquitin-activating enzyme (E1), the proteasome activators PA700 and PA28, and Hsp70, all of which enhance the proteolysis rate [174]. The authors suggest on the basis of these data that Lewy body formation in the brain of patients suffering from Parkinson's disease is similar to aggresome-like structure formation under conditions when proteasome capacity is exceeded.

Neurodegenerative polyglutamine diseases characterized by disorder of motor coordination, such as Huntington's disease and Machado–Joseph disease are caused by the same type of mutations. As a result of the genetic failure, cells begin to synthesize irregular proteins with glutamine repeat expansion. Development of Huntington's disease is associated with formation of intranuclear inclusions in neurons containing accumulations of polyglutamine fragments of mutant huntingtin [179-181]. The normal form of huntingtin contains no more than 20 glutamine repeats, whereas the mutant protein contains more than 40 glutamine residues and possesses a higher ability to aggregate. Intranuclear huntingtin aggregates, containing amyloid-like fibrils, formed

due to self-assembly of β-sheets, are toxic, can block transcription factors, inhibit UPS, and cause neuronal death [182]. It has been suggested that fragments of mutant and wild-type huntingtin can co-aggregate in neurons of patients with Huntington's disease and contribute to the pathogenesis of the disease [7]. However, besides intranuclear inclusions, as has been demonstrated on human 293 Tet-Off cells, mutant huntingtin containing 51 or 83 glutamine repeats instead of 20 repeats in the normal range, form aggresome-like perinuclear inclusions [183]. These structures contain mutant form of aggregated, ubiquitinated huntingtin with a characteristic fibrillar structure. Inclusion bodies with truncated protein are localized at centrosomes and are surrounded by vimentin filaments. Immunofluorescence and electron microscopy revealed that subunits of 26S proteasome, molecular chaperones BiP/GRP78, Hsp70, and Hsp40 and α-synuclein are co-localized within the perinuclear inclusions. Formation of perinuclear aggregates containing expanded polyglutamine stretches, which are co-localized with γ-tubulin and surrounded by vimentin filaments, has also been demonstrated on COS-7 cells [184]. Disruption of the microtubules by nocodazole results in the formation of small aggregates, which are scattered throughout the cytoplasm. It is suggested that proteins containing expanded polyglutamine stretches are unfolded and form aggregates, which move along microtubules to the MTOC, forming aggresomes [154].

Formation of mutant forms of intracellular prion protein (PrP) underlies the pathogenesis of prion diseases—infectious, inherited, or sporadic human and animal neurodegenerative disorders [9, 185, 186]. The best known of these are Creutzfeldt–Jakob disease and Gerstmann–Straussler–Scheinker syndrome of humans, scrapie of sheep, and spongioform encephalopathy of bovines, sometimes called prurigo of sheep, mad-cow disease, or spongioform encephalitis. These diseases are accompanied by morphological alterations of the brain tissues and by appearance of amyloid fibrils containing prion protein. Native PrP is a proline-rich glycoprotein bound to the cell membrane [95]. PrP is characterized by quite a high content of α-helices and low content of β-structure, is soluble in detergents, sensitive to the action of proteinases, and does not form aggregates [187, 188]. Several mutant forms of prion proteins (PrP V203I and E211Q) causing the Creutzfeldt–Jakob disease and PrP Q212P typical for the Gerstmann–Straussler–Scheinker syndrome are known [10]. The best studied of these is the mutant infective form of PrPSc protein associated with infectional spongioform encephalopathy [189]. PrPSc is produced as a result of polypeptide misfolding, predominantly possesses β-folded structure, and is detergent-insoluble and stable to the action of proteinases [95, 188, 190]. β-Sheet containing proteins form amyloid-like fibrils [191]. It has been demonstrated that transfection of human neuroblastoma cells NT-2 by plasmid DNA, responsible for encoding PrP, and incubation of cells in the presence of proteasome inhibitor are accompanied by accumulation of aggregates of all three prion mutants, which often include Hsc70 chaperone. On prolonged incubation, the aggregates localize around the centrosome forming aggresome-like structures [10, 186].

Formation of high-molecular-weight insoluble complexes of aggregated mutant superoxide dismutase (SOD) causes familial paralytic disease—amyotrophic lateral sclerosis accompanied by degeneration of motor neurons in cortex, brainstem, and spinal cord [4, 192, 193]. Mutant forms of SOD in *in vitro* studies demonstrate increased ability to form aggregates [4, 194]. It has been suggested that increase in the ability of SOD to aggregate can occur due to increase in the share of unfolded or partially folded forms in the population of molecules that are in a state of equilibrium, due to increase in unfolding rate or, finally, due to decrease in folding rate. It follows from available data that increase in toxicity of various mutant SOD forms, causing amyotrophic lateral sclerosis, is a result of enzyme destabilization that leads to increase in the amount of cytotoxic aggregates. *In vivo* experiments demonstrate that formation in spinal cord of transgenic mice of SOD aggregates, which are detectable in spinal cord extracts, completes by formation of inclusion bodies and pathologic alterations in motor neurons [10]. Moreover, it has been revealed that deposition of mutant SOD in the cytoplasmic inclusion bodies, similar to aggresomes, proceeds due to microtubule-dependent transport of aggregates [10, 14].

Despite the differences in the pathologic disorders accompanying the above-described neurodegenerative diseases, all of them involve formation of protein aggregates and degeneration of specific nerve cells. However, causal relationship between protein aggregation and neurodegeneration has not been established to date. It is generally accepted that protein aggregation takes a central place in cell death [195], yet it remains unclear whether protein aggregates cause or are a consequence of neurotoxicity.

It follows from experimental data available in the literature that aggregated proteins impair UPS function [196]. UPS inhibition in cells containing protein aggregates may underlie pathogenesis of many neurodegenerative disorders [149]. Expression of two unrelated proteins, a huntingtin fragment containing polyglutamine repeat and CFTR, whose only common property is predisposition to misfolding and aggregation, causes nearly complete UPS inhibition. Aggregation of these proteins leads to accumulation of intracellular ubiquitin conjugates. Because of the central role of ubiquitin-dependent proteolysis in regulation of cellular events such as cell division and apoptosis, a mechanism connecting protein aggregation with cellular disregulation and cell death has been suggested [196]. Since no decrease in free ubiquitin in cells containing inclusion bodies is revealed, it is suggest-

ed that protein aggregates can inhibit UPS by saturation of one or more chaperones (especially Hsc70) essential to operation of this system [197], or by direct interaction with proteasomes. Reduction of UPS activity in damaged neurons may be a result of protein aggregate formation and may be responsible for production of ubiquitin conjugates [198] and of UPS substrates [199]. Increase in aggregation level can lead to subsequent decrease in UPS function. Existence of a such feedback mechanism might explain a sharp decrease of the functional activity of neurons that is observed in many neurodegenerative disorders.

UNIVERSALITY OF AGGRESOME FORMATION

The mechanism of cellular defense against toxic protein aggregates may involve movement of aggregated proteins in cells in the direction of inclusion bodies or aggresomes [14, 73, 155, 159]. Aggresome formation is a general cellular response to the presence of misfolded proteins when a cell's capacity to degrade them has been exceeded [14, 73, 77, 155, 159]. In addition to aggresome formation in cells expressing integral membrane proteins CFTR and PS1 [14], formation of aggresomes and their pericentriolar localization are also observed in the case of aggregated forms of other misfolded or incorrectly assembled integral membrane proteins, including periphery myelin protein PMP22 [200], as well as misfolded cytoplasmic proteins: chimera protein, obtained from GFP and a fragment of protein p115 [77], and a mutant form of SOD [10]. A mutant form of surfactant protein C, a 35-amino acid hydrophobic lung-specific protein produced exclusively by alveolar type 2, also possesses an ability to form aggresomes [201-204].

Thus, aggresomes have been revealed in cells expressing CFTR, presenilin-1, mutant protein GFP-250, mutant forms of huntingtin, SOD, prion proteins, α-synuclein, parkin, and mutant protein proSPC when these proteins are over-expressed or when the cells are treated with compounds which inhibit proteasome activity. It has been established that mature aggresomes may connect with lysosomes, and as a result, the proteins incorporated in these inclusions are degraded with higher rate. Reasoning from the above, it is suggested that aggresomes, acting as protein-deposition centers, perform a defense role in the cell, while accelerating the process of degradation of toxic proteins [154]. It is conceivable that pathologies originating in the presence of aggregated proteins in cells are not a consequence of aggresome formation, but, more likely, arise in the absence of functional activity of aggregated proteins and/or due to toxicity of protein aggregates.

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