Chapter 7

Cellular Stress and Protein Misfolding During Aging

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Abstract

Cells are under constant onslaught from several intrinsic and extrinsic stressors, which lead to the occurrence and accumulation of molecular damage, functional impairment, aging, and eventual death. Protein misfolding is both a cause and a consequence of increased cellular stress. An age-related failure of the complex systems for handling protein misfolding results in the accumulation of misfolded and aggregated proteins, and consequent conformational diseases. However, some misfolded proteins have been found to be both toxic and, in some cases, protective, highlighting the various complex, dynamic, and interdependent mechanisms at play. Molecular mechanisms are being elucidated for the occurrence of protein misfolding and for its prevention by chaperones and various pathways of degradation. Insights from the knowledge about proteodynamics are likely to impact future interventional strategies to counter stress and to promote healthy aging by preventing and/or treatment of protein conformational diseases.

Key words: Abnormal proteins, Protein turnover, Proteasome, Lysosome, Proteostasis, Proteodynamics

1. Introduction

Aging at the molecular level is characterised by the progressive accumulation of molecular damage in DNA, RNA, lipids, and proteins (1). An age-related increase in the levels of structurally and functionally abnormal proteins is a universally observed phenomenon (2, 3). A variety of stressors have been implicated in the occurrence and increase of abnormal proteins during aging, of which oxidative stress is a major contributor. Chronic oxidative stress can lead to protein misfolding or unfolding, resulting in proteins unable to carry out their normal functions and thus initiating a breakdown of various cellular events. Mammalian cells have various maintenance, repair, and removal systems to counteract such events, and include the GroEL chaperone pathway to refold proteins (4), the ubiquitin-mediated proteosomal degradational

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machinery to remove misfolded proteins (5), and lysosomal autophagy to sequester and degrade larger aggregates (6). Inefficient protein turnover by both proteasomal and lysosomal pathways are generally considered to be the main reason for the accumulation of abnormal proteins during aging (1). Recently, however, protein misfolding and its deleterious consequences have emerged as the crucial mediators of impaired cellular functions and increased cellular stress during aging. This is also underscored by observations that resistance to protein folding damage and proper protein folding over time correlate with species-specific longevity (7, 8). Thus, tight control of protein folding and misfolding seems to be the key in regulating cellular stress during aging.

It is well recognised that properly folded proteins are necessary for their efficient and accurate functions. Since the first demonstration by C. Anfinsen in 1973 that it is the primary amino acid sequence that determines the final protein structure (9), it is now well accepted that protein folding in vitro is many times simpler than what is known to happen in vivo. A remarkable fact in protein folding is the observation that proteins can fold in the millisecond timescale in a complex environment in which other proteins and macromolecules like DNA, RNA, and lipids populate the cellular space in concentration ranges of 300–400 mg/ml (10). This amazing biological feat clearly points out that protein folding is not an isolated event but is a "team event" with a number of molecules within this crowded interior assisting in the formation of the final correctly folded product. Thus, to attain proper function in the complex environment of a cell, appropriate mechanisms have evolved for folding proteins in the "right way," which overall comprise what is also known as the protein "quality control system."

The term "misfolded" proteins refers both to proteins that are unable to form a well-structured compact fold and to proteins that have attained a compact fold but a wrong one with the wrong amino acids resulting in aberrant or promiscuous functionality. What the term does not refer to is a new class of proteins that have been recently identified as functional proteins lacking a compact fold – the so-called "unfolded proteome" (11, 12). In this article, we review the basic mechanisms governing the process of protein folding, the occurrence of misfolding, and how it contributes towards increasing cellular stress during aging.

2. Protein Folding: A Team Event

Like all biological processes, protein folding requires energy, and is a mechanism that follows the basic thermodynamic principles. It has also been demonstrated that the folding process searches

for the structural conformation with the lowest energy (13), again reaffirming Anfinsen's principle that the folding code is contained within the sequence itself. While Anfinsen's conclusions still hold for the most part, it has also become clear that the complex environment of a cell contains other "players," which are involved in the formation of the final, functional, and low-energy structure.

Many such macromolecules identified in this team-play are chaperones and folding catalysts. Molecular chaperones are the molecules whose primary function is to guide proteins to their "proper" fate but not to remain associated with the final "product" – a fully folded and functional protein (14). In comparison, folding catalysts are the molecules that accelerate the steps within the folding process, for example, disulphide isomerases which enhance the rate of formation and reorganisation of disulphide bonds within proteins (15). First set of evidence that chaperones prevent misfolding came from the observations that their levels were significantly increased under stress conditions such as heat, which cause protein denaturation and misfolding (16). Indeed, it is the chaperones that have emerged as the single largest class of molecules with an important role in the protein folding pathway, and an increasing diversity of their functions is still being uncovered. Not only do these molecules ensure that the proteins attain their native state, but also complex mechanisms have evolved to assist the folding of newly synthesised polypeptides and rescue existing proteins from partial stress-induced denaturation (17).

2.1. The Backstage Players: Multimodal Chaperones

A correctly folded protein is essential for carrying out functions within a cell. The mechanisms by which chaperones act differ depending on their nature and on their cellular location, but their active intervention in rescuing misfolded proteins is an energy consumptive process, and is thus largely driven by ATP (18). The GroEL-GroES chaperone system, for example, uses ATP to bind and release unfolded polypeptides (4). The binding stabilises the substrate in an unfolded state until its subsequent folding and release. The GroEL chaperone complex has been proposed to assist in protein folding via the Anfinsen Cage model (19, 20), whereby the cavity of the complex provides a stable microenvironment in which protein folding can occur unhindered and protected from aggregation. Another class of chaperones, the DnaK (Hsp70) family binds to unfolded proteins via exposed hydrophobic residues, thereby preventing misfolding and subsequent aggregation (21). The folding is then completed in the cytoplasm upon their release. In both cases, the chaperones partake indirectly without providing any folding information to the substrate.

An additional, albeit contrasting, function of chaperones has been found in the small periplasmic PapD-like chaperone family where protein information is supplied transiently to their substrate. This function was first observed in the subunit proteins of *E. coli*

responsible for building up surface adhesive organelles whereby the chaperones supply steric information to their substrate proteins (22). An example of this is seen in the pilus subunits of gram-negative bacteria where PapD-like chaperones (of which more than 30 members exist in this superfamily) actually donate a beta strand of their own to the subunit to stabilise the complex (23). Interestingly, in the absence of the chaperone, a protease DegP, which recognises misfolded, denatured, and/or aggregated proteins, takes over, and degrades the now unstable subunits. DegP in itself has recently been shown not to be just a protease but more of a protease-chaperone. It has been observed that the binding of misfolded proteins transforms hexameric DegP into large active 12- or 24-mers where the highly flexible inner cavity serves opposite functions depending on the substrate (24), where outer membrane proteins are provided a safe transit while misfolded proteins are degraded within the same interior.

3. Stress and Protein Misfolding

Cells are under constant onslaught from several intrinsic and extrinsic stressors, which range from physical and metabolic stressors to environmental and genetic stressors. These also include changes in the microenvironment of the cell, up- or down-regulation of concentrations of metabolites and/or osmolytes (that can induce protein stability changes), temperature changes, pH changes, and so on. The consequent protein misfolding, if not rescued in time, can result in aberrant aggregate formation, whose accumulation can eventually lead to the onset of a disease phenotype. How cellular stress and protein misfolding overwhelm the protein quality control mechanisms to cause disease in the context of aging is discussed below.

3.1. Protein Homeodynamics in a Cell

"Proteostasis" has been suggested as a term to describe the control of the protein concentration, conformation, binding interactions, and cellular location by readapting the internal biology of the cell mostly through transcriptional and translational changes (25). Since the above processes are generally very dynamic, it may be more appropriate to call it as "proteodynamics," in line with increasing replacement of the term "homeostasis" with "homeodynamics" in biological systems (26). Indeed the protein quality control mechanisms within a cell, utilising various assistants in the protein folding pathway and other competing degradation pathways, indicate that proteodynamics plays a critical role in the cell. Evidence for the importance of the quality control is also highlighted by the fact that up to half of all the polypeptide chains fail to satisfy the quality control mechanism in the endoplasmic reticulum

(ER), and for some proteins the success rate is even lower (27). Like the HSP-response in the cytoplasm, the "unfolded protein response" (UPR) (28) in the ER is also up-regulated during stress and is strongly linked to the avoidance of protein misfolding diseases.

3.2. Natively Unfolded Proteins

Another recently observed parallel pathway in cells is that of the "natively unfolded proteome" (29). As the name suggests, these proteins are natively unstructured but are still functional, and a rather significant part of the eukaryotic genome actually codes for such proteins. The discovery of these proteins has challenged the paradigm of the need of a compact, ordered, and three-dimensional protein structure to carry out the function (30). Many of these proteins remain permanently unstructured under physiological conditions whereas many others interact with binding partners and then fold into a functional form making them ideal players in multiple targeting and cellular regulation. The presence of the natively unfolded proteome also suggests that chaperones and other macromolecular assistants probably bind to these proteins also and keep them from getting aggregated. One of the largest class of such natively unfolded proteins are the synucleins (31), of which the most extensively studied protein is α-synuclein for its role in Parkinson's disease (32). The cellular co-existence of parallel and seemingly antagonistic proteomes – the folded and unfolded proteome – is a clear example of proteodynamics in the cell. Imbalances in the proteodynamics, which could result from misfolding or aggregation, are sure to lead to functional impairment, disregulation, and other consequences including cell death, aging, and diseases.

3.3. Protein Misfolding and Aggregation as a Function of Stress

There are various factors in a cell – both intrinsic and extrinsic – that can raise cellular stress levels, and a cumulative action of these may overwhelm the quality control mechanisms, and result in the misfolding and/or aggregation of proteins. The main factors involved are spontaneous mutations due to intrinsic errors in DNA duplication, induced mutations due to reactive oxygen species (ROS), and other free radicals, errors in protein synthesis, and post-translational modifications due to damage by ROS, by other free radicals, and by nutritional metabolites such as glyoxal and methylglyoxal.

The role of mutations in protein misfolding is clearly identified and, as previously mentioned, this can be a severe form of stress in the cell. However, not all mutations have deleterious effects and thus it is important to distinguish the different types of mutations that can act as cellular stressors and those that cannot. Classification of mutations per se can be very complex as there may be many ways to define them based on pattern of inheritance, effect on the primary structure, phenotype, and function,

and more importantly by their impact on protein sequence. However, generally mutations are classified based on their effect on the primary structure, and as such have three main types – point mutations, deletions, and insertions. Point mutations can be further subdivided into: (i) silent mutations – which code for similar amino acids; (ii) missense mutations – which code for different amino acids; and (iii) nonsense mutations – which code for a stop codon and can result in a truncated gene product.

For large deletions and, in most cases, stop mutations, the deleterious effect is obvious as the function of the affected polypeptide product is abolished. However, for missense mutations or short in-frame insertions and deletions, the disease-causing nature of the mutation is not directly evident (33). The Human Gene Mutation Database contains, at present, close to 88,000 entries on mutations in genetic diseases, of which approximately 49,000 entries are missense mutations (34). Missense mutations and short in-frame deletions and insertions often impair the propensity of the affected polypeptide to fold to the functional conformation, and/or decrease the stability of the functional conformation. Both effects lead to an increase in the proportion of mutant polypeptide present in nonfunctional conformations that are more susceptible to degradation or aggregation than the functional conformation.

Thus, diseases arising from misfolded or non-functional conformations were termed as conformational diseases (35), referring to conditions in which aggregation due to aberrant folding of a polypeptide appears to be the molecular pathological mechanism, as for example in Alzheimer's disease (AD) and Creutzfeldt-Jakob's disease. However, in a wider definition (36), conformational diseases were linked to disturbances of the folding process in general (37), thereby distinguishing two subgroups: one covered by the appearance of aggregating proteins and a second group of diseases in which impaired folding leads to rapid degradation of the affected polypeptides (e.g., cystic fibrosis and most forms of α-1-antitrypsin deficiency).

Over the years, a large number of human diseases have been linked to conformational upheavals or misfolding. The involvement of mutations in these diseases has also resulted in an alternate classification of these diseases by their effect on function. Inherited disorders like cystic fibrosis are due to amino acid substitutions that exhibit a loss-of-function pathogenesis because the aberrant protein is eliminated by the protein quality control system in the ER (38). However, not all aberrant proteins can be eliminated and the misfolded protein may accumulate and form toxic oligomeric and/or aggregated inclusions. In this case, the loss of function of a protein may be accompanied by a gain-of-function pathogenesis, which in many cases determines the pathological and clinical features, examples being Parkinson's and

Table 1
Representative protein misfolding diseases showing the proteins involved, the cellular location in which they fold, and the basic molecular pathogenesis

Protein involved	Location	Pathogenic mode	Disease
β-amyloid	C/E	Gain-of-function	Alzheimer's disease
Crystallin	C	Gain-of-function	Cataract
CFTR	ER	Loss-of-function	Cystic Fibrosis
Prion	ER	Loss-of-function	Creutzfeldt–Jakob disease or CJD
α-1-antitrypsin	ER	Loss-of-function	α-1-antitrypsin deficiency
Transthyretin (TTR)/ Lysozyme	ER	Gain-of-function	Familial amyloidosis
SCAD variants	M	Loss-of-function	Short-chain acyl-CoA dehydrogenase (SCAD) deficiency
LDL receptor	ER	Loss-of-function	Familial hypercholesterolemia

C cytoplasm, *E* extracellular, *ER* endoplasmic reticulum, *M* mitochondria

Huntington's disease. Table 1 lists some of the known protein misfolding or protein conformational diseases, the proteins involved in such disorders, their cellular location, and the suggested molecular pathogenesis.

3.4. Errors and Damage in Proteins

Since, the error frequency of amino acid misincorporation is generally considered to be quite high (3) as compared with nucleotide misincorporation (less than 10^{-6}), the role of protein error feedback in aging has been a widely discussed issue, and is the basis of the so-called error catastrophe theory of aging (3, 39). So far, no direct estimates of protein error levels in any aging system have been made, primarily due to the lack of appropriate methods to determine spontaneous levels of errors in a normal situation. However, several indirect estimates of the accuracy of translation in cell-free extracts, using synthetic templates or natural mRNAs have been made. Studies performed on various young and old animal tissues such as chick brain, mouse liver, and rat brain, liver, and kidney, and human cells undergoing aging in vitro did reveal some age-related increase in protein errors (3, 39). Furthermore, an induction and increase in protein errors has been shown to accelerate aging in human cells and bacteria (1, 40). It will be important to know if there is a direct link between increased protein errors and increased protein misfolding during aging.

A large number of post-translational modifications of proteins have been described that determine the activity, stability, specificity, transportability, and lifespan of a protein. Several of these modifications are highly specific and regulated involving various

enzymatic pathways, for example, phosphorylation, methylation, ADP-ribosylation, glycosylation, and acetylation. However, there are several non-enzymatic modifications of proteins, which occur stochastically and are considered as damage, for example, oxidation, glycation, and racemization. Many of these alterations in proteins will lead to protein misfolding and to consequent cellular stress and aging. A large number of oxidatively damaged proteins have been reported to accumulate during aging in a wide variety of biological systems including cells, tissues, organs, and organisms (1, 40).

4. Aging, Diseases, and Protein Misfolding

The past decade has seen an explosion in the literature on misfolding diseases in general, and it has become evident that the largest group of misfolding diseases are those associated with the conversion of proteins into highly organised fibrillar aggregates. These structures are known as amyloid fibrils or plaques when referring to extracellular deposits, and as "intracellular inclusions" when observed inside the cell (41). Owing to the fact that these aggregates usually involve the formation of tissue deposits, either extracellular or intracellular, the diseases represented by them are also known as protein deposition diseases.

Whether all such deposits are harmful (42, 43) or may be even protective is still being debated (44), as both toxic and non-toxic effects have been reported (45, 46). Such a phenomenon of protective effects of potentially harmful conditions is also known as hormesis (47). A hitherto unexplored hypothesis has also been put forward that the cell itself may be targeting the protein to form non-toxic aggregates as a form of defence mechanism (48). This has indeed been seen in *E. coli* (48) where it was suggested that the function of protein aggregates is a type of "trash organelle" for cellular detoxification. While one may wonder at the cause and effect pattern of protein aggregates, it is clear that the presence and number of aggregates is heavily biased towards an aged cell (49). However, this is a result of damage that has started when the cell was young, and the toxic cascade was already at work during an apparently healthy looking cell. Indeed, it has been clearly shown that in many misfolding or deposition diseases, soluble oligomers (low molecular weight aggregates) are the major culprits responsible for toxicity (50) in contrast to higher molecular weight aggregates that do not correlate with toxicity (45). All these studies thus show that misfolding diseases and protein deposition diseases are two sides of the same coin. Furthermore, it has been shown that the same protein under pathological conditions can lead to the formation of fibrillar,

pore-like, spherical, or amorphous aggregates with diverse biological consequences (51), but the conditions leading to misfolding and the formation of such abnormal complexes are unclear. The mechanisms underlying aggregation in vivo in biological systems are even less clearly understood due to the experimental difficulties in monitoring aggregates in their natural environment.

To summarise, a schematic representation of the occurrence and consequences of protein misfolding is given in Fig. 1. While accurate protein synthesis, followed by correct protein folding, is essential for the normal functioning of proteins, transcriptional and translational errors or stressful conditions can result in protein misfolding. Unless misfolding is counteracted by either chaperone-mediated refolding processes or proteasome-lysosome-mediated protein degradation, misfolded proteins can form aggregates with varying consequences. Whereas the so-called non-toxic aggregates may even have some beneficial hormetic effects by challenging the homeodynamic processes, large and toxic aggregates increase intracellular stress levels and consequent impairment in function, including aging, diseases, and death. With a deeper understanding of these phenomena, there is a hope for the development of efficient therapeutics capable of preventing or reversing the occurrence of protein misfolding and deposition diseases during the life-time of an individual.

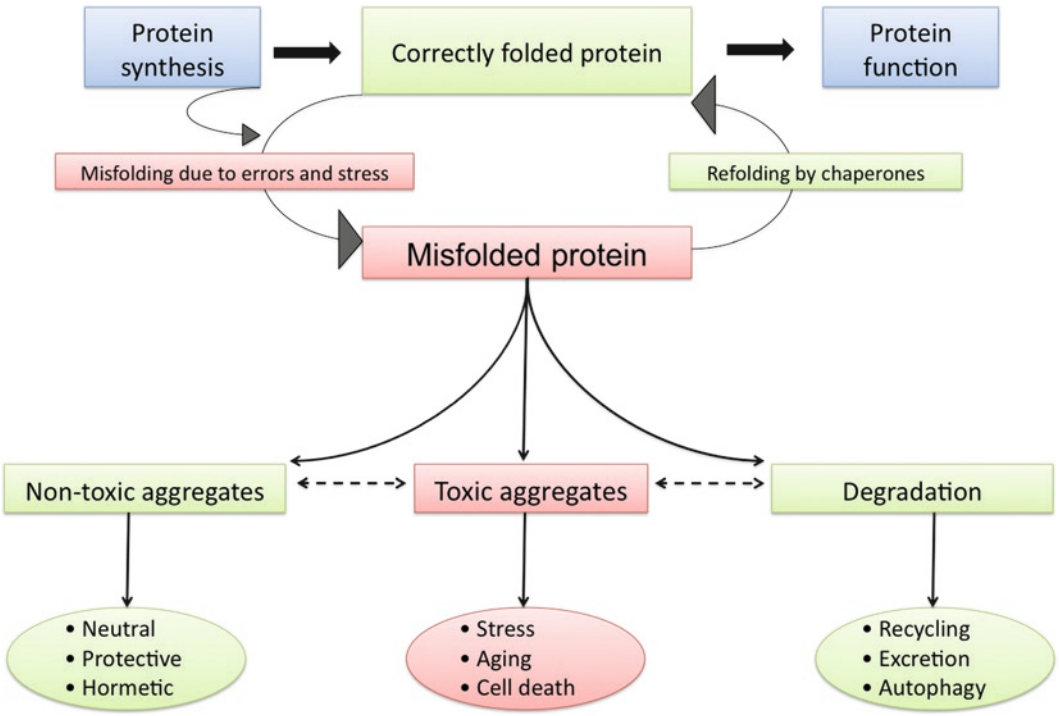

Fig. 1. Schematic representation of the occurrence of protein misfolding and the consequences of failed defences.

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