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INVITED REVIEW

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Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution

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Abstract The deposition of proteins in the form of amyloid fibrils and plaques is the characteristic feature of more than 20 degenerative conditions affecting either the central nervous system or a variety of peripheral tissues. As these conditions include Alzheimer's, Parkinson's and the prion diseases, several forms of fatal systemic amyloidosis, and at least one condition associated with medical intervention (haemodialysis), they are of enormous importance in the context of present-day human health and welfare. Much remains to be learned about the mechanism by which the proteins associated with these diseases aggregate and form amyloid structures, and how the latter affect the functions of the organs with which they are associated. A great deal of information concerning these diseases has emerged, however, during the past 5 years, much of it causing a number of fundamental assumptions about the amyloid diseases to be re-examined. For example, it is now apparent that the ability to form amyloid structures is not an unusual feature of the small number of proteins associated with these diseases but is instead a general property of polypeptide chains. It has also been found recently that aggregates of proteins not associated with amyloid diseases can impair the ability of cells to function to a similar extent as aggregates of proteins linked with specific neurodegenerative conditions. Moreover, the mature amyloid fibrils or plaques appear to be substantially less toxic than the prefibrillar aggregates that are their precursors. The toxicity of these early aggregates appears to result from an intrinsic ability to impair fundamental cellular processes by interacting with cellular membranes, causing oxidative stress and increases in free Ca^{2+} that eventually lead to apoptotic or necrotic cell death. The 'new view' of these diseases also suggests that other degenerative conditions could have similar underlying origins to those of the amyloidoses. In addition, cellular protection mechanisms, such as molecular chaperones and the protein degradation machinery, appear to be crucial in the prevention of disease in normally functioning living organisms. It also suggests some intriguing new factors that could be of great significance in the evolution of biological molecules and the mechanisms that regulate their behaviour.

Keywords Amyloid aggregates · Amyloidoses · Molecular evolution · Folding and disease · Protein aggregation

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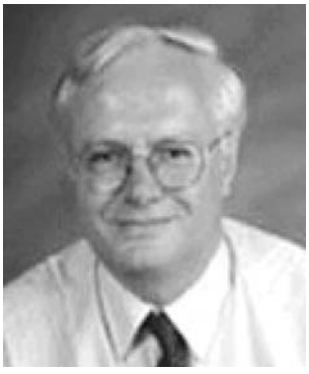

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Abbreviations *ER:* Endoplasmic reticulum · *Hsp:* Heat-shock protein · *HypF-N:* N-terminal domain of hydrogenase maturation factor HypF · *ROS:* Reactive oxygen species · *SH3:* Src-homology 3

Introduction

The genetic information within a cell encodes not only the specific structures and functions of proteins but also the way these structures are attained through the process known as protein folding. In recent years many of the underlying features of the fundamental mechanism of this complex process and the manner in which it is regulated in living systems have emerged from a combination of experimental and theoretical studies [1]. The knowledge gained from these studies has also raised a host of interesting issues. It has become apparent, for example, that the folding and unfolding of proteins is associated with a whole range of cellular processes from the trafficking of molecules to specific organelles to the regulation of the cell cycle and the immune response. Such observations led to the inevitable conclusion that the failure to fold correctly, or to remain correctly folded, gives rise to many different types of biological malfunctions and hence to many different forms of disease [2]. In addition, it has been recognised recently that a large number of eukaryotic genes code for proteins that appear to be 'natively unfolded', and that proteins can adopt, under certain circumstances, highly organised multi-molecular assemblies whose structures are not specifically encoded in the amino acid sequence. Both these observations have raised challenging questions about one of the most fundamental principles of biology: the close relationship between the sequence, structure and function of proteins, as we discuss below [3].

It is well established that proteins that are 'misfolded', i.e. that are not in their functionally relevant conformation, are devoid of normal biological activity. In addition, they often aggregate and/or interact inappropriately with other cellular components leading to impairment of cell viability and eventually to cell death. Many diseases, often known as misfolding or conformational diseases, ultimately result from the presence in a living system of protein molecules with structures that are 'incorrect', i.e. that differ from those in normally functioning organisms [4]. Such diseases include conditions in which a specific protein, or protein complex, fails to fold correctly (e.g. cystic fibrosis, Marfan syndrome, amyotonic lateral sclerosis) or is not sufficiently stable to perform its normal function (e.g. many forms of cancer). They also include conditions in which aberrant folding behaviour results in the failure of a protein to be correctly trafficked (e.g. familial hypercholesterolaemia, α_1-antitrypsin deficiency, and some forms of retinitis pigmentosa) [4]. The tendency of proteins to aggregate, often to give species extremely intractable to dissolution and refolding, is of course also well known in other circumstances. Examples include the formation of inclusion bodies during overexpression of heterologous proteins in bacteria and the precipitation of proteins during laboratory purification procedures. Indeed, protein aggregation is well established as one of the major difficulties associated with the production and handling of proteins in the biotechnology and pharmaceutical industries [5].

Considerable attention is presently focused on a group of protein folding diseases known as amyloidoses. In these diseases specific peptides or proteins fail to fold or to remain correctly folded and then aggregate (often with other components) so as to give rise to 'amyloid' deposits in tissue. Amyloid structures can be recognised because they possess a series of specific tinctorial and biophysical characteristics that reflect a common core structure based on the presence of highly organised β-sheets [6]. The deposits in strictly defined amyloidoses are extracellular and can often be observed as thread-like fibrillar structures, sometimes assembled further into larger aggregates or plaques. These diseases include a range of sporadic, familial or transmissible degenerative diseases, some of which affect the brain and the central nervous system (e.g. Alzheimer's and Creutzfeldt-Jakob diseases), while others involve peripheral tissues and organs such as the liver, heart and spleen (e.g. systemic amyloidoses and type II diabetes) [7, 8]. In other forms of amyloidosis, such as primary or secondary systemic amyloidoses, proteinaceous deposits are found in skeletal tissue and joints (e.g. haemodialysis-related amyloidosis) as well as in several organs (e.g. heart and kidney). Yet other components such as collagen, glycosaminoglycans and proteins (e.g. serum amyloid protein) are often present in the deposits protecting them against degradation [9, 10, 11]. Similar deposits to those in the amyloidoses are, however, found intracellularly in other diseases; these can be localised either in the cytoplasm, in the form of specialised aggregates known as aggresomes or as Lewy or Russell bodies or in the nucleus (see below).

The presence in tissue of proteinaceous deposits is a hallmark of all these diseases, suggesting a causative link between aggregate formation and pathological symptoms (often known as the amyloid hypothesis) [7, 8, 12]. At the present time the link between amyloid formation and disease is widely accepted on the basis of a large number of biochemical and genetic studies. The specific nature of the pathogenic species, and the molecular basis of their ability to damage cells, are however, the subject of intense debate [13, 14, 15, 16, 17, 18, 19, 20]. In neurodegenerative disorders it is very likely that the impairment of cellular function follows directly from the interactions of the aggregated proteins with cellular components [21, 22]. In the systemic non-neurological diseases, however, it is widely believed that the accumulation in vital organs of large amounts of amyloid deposits can by itself cause at least some of the clinical symptoms [23]. It is quite possible, however, that there are other more specific effects of aggregates on biochemical processes even in these diseases. The presence of extracellular or intracellular aggregates of a specific polypep-

Table 1 A summary of the main amyloidoses and the proteins or peptides involved

Disease	Main aggregate component
Alzheimer's disease	Aβ peptides (plaques); tau protein (tangles)
Spongiform encephalopathies	Prion (whole or fragments)
Parkinson's disease	α-synuclein (wt or mutant)
Primary systemic amyloidosis	Ig light chains (whole or fragments)
Secondary systemic amyloidosis	Serum amyloid A (whole or 76-residue fragment)
Fronto-temporal dementias	Tau (wt or mutant)
Senile systemic amyloidosis	Transthyretin (whole or fragments)
Familial amyloid polyneuropathy I	Transthyretin (over 45 mutants)
Hereditary cerebral amyloid angiopathy	Cystatin C (minus a 10-residue fragment)
Haemodialysis-related amyloidosis	β_2-microglobulin
Familial amyloid polyneuropathy III	Apolipoprotein AI (fragments)
Finnish hereditary systemic amyloidosis	Gelsolin (71 amino acid fragment)
Type II diabetes	Amylin (fragment)
Medullary carcinoma of the thyroid	Calcitonin (fragment)
Atrial amyloidosis	Atrial natriuretic factor
Hereditary non-neuropathic systemic amyloidosis	Lysozyme (whole or fragments)
Injection-localised amyloidosis	Insulin
Hereditary renal amyloidosis	Fibrinogen α-A chain, transthyretin, apolipoprotein AI, apolipoprotein AII, lysozyme, gelsolin, cystatin C
Amyotrophic lateral sclerosis	Superoxide dismutase 1 (wt or mutant)
Huntington's disease	Huntingtin
Spinal and bulbar muscular atrophy	Androgen receptor [whole or poly(Q) fragments]
Spinocerebellar ataxias	Ataxins [whole or poly(Q) fragments]
Spinocerebellar ataxia 17	TATA box-binding protein [whole or poly(Q) fragments]

tide molecule is a characteristic of all the 20 or so recognised amyloid diseases. The polypeptides involved include full length proteins (e.g. lysozyme or immunoglobulin light chains), biological peptides (amylin, atrial natriuretic factor) and fragments of larger proteins produced as a result of specific processing (e.g. the Alzheimer β-peptide) or of more general degradation [e.g. poly(Q) stretches cleaved from proteins with poly(Q) extensions such as huntingtin, ataxins and the androgen receptor]. The peptides and proteins associated with known amyloid diseases are listed in Table 1. In some cases the proteins involved have wild type sequences, as in sporadic forms of the diseases, but in other cases these are variants resulting from genetic mutations associated with familial forms of the diseases. In some cases both sporadic and familial diseases are associated with a given protein; in this case the mutational variants are usually associated with early-onset forms of the disease. In the case of the neurodegenerative diseases associated with the prion protein some forms of the diseases are transmissible. The existence of familial forms of a number of amyloid diseases has provided significant clues to the origins of the pathologies. For example, there are increasingly strong links between the age at onset of familial forms of disease and the effects of the mutations involved on the propensity of the affected proteins to aggregate in vitro. Such findings also support the link between the process of aggregation and the clinical manifestations of disease [24, 25].

The presence in cells of misfolded or aggregated proteins triggers a complex biological response. In the cytosol, this is referred to as the 'heat shock response' and in the endoplasmic reticulum (ER) it is known as the 'unfolded protein response'. These responses lead to the expression, among others, of the genes for heat shock proteins (Hsp, or molecular chaperone proteins) and proteins involved in the ubiquitin-proteasome pathway [26]. The evolution of such complex biochemical machinery testifies to the fact that it is necessary for cells to isolate and clear rapidly and efficiently any unfolded or incorrectly folded protein as soon as it appears. In itself this fact suggests that these species could have a generally adverse effect on cellular components and cell viability. Indeed, it was a major step forward in understanding many aspects of cell biology when it was recognised that proteins previously associated only with stress, such as heat shock, are in fact crucial in the normal functioning of living systems. This advance, for example, led to the discovery of the role of molecular chaperones in protein folding and in the normal 'housekeeping' processes that are inherent in healthy cells [27, 28]. More recently a number of degenerative diseases, both neurological and systemic, have been linked to, or shown to be affected by, impairment of the ubiquitin-proteasome pathway (Table 2). The diseases are primarily associated with a reduction in either the expression or the biological activity of Hsps, ubiquitin, ubiquitinating or deubiquitinating enzymes and the proteasome itself, as we show below [29, 30, 31, 32], or even to the failure of the quality control mechanisms that ensure proper maturation of proteins in the ER. The latter normally leads to degradation of a significant proportion of polypeptide chains before they have attained their native conformations through retrograde translocation to the cytosol [33, 34]. For example, the most common mutation of the CFTR chloride channel associated with cystic fibrosis interferes with the cor-

Table 2 Neurodegenerative diseases with inclusion bodies shown to be linked to deficits of the ubiquitin-proteasome pathway (modified from [26])

Disease	Main fibril constituent
Alzheimer's disease	Tau
Fronto-temporal dementia	Tau
Parkinson's disease	α-Synuclein/crystallin
Dementia with Lewy body	α-Synuclein
Amyotrophic lateral sclerosis	Superoxide dismutase
Poly-Q extension disorders	
Huntington's disease	Huntingtin
Spinocerebellar ataxias	Ataxins 1, 2 and 3
Spinobulbar muscular atrophy (Kennedy's)	Androgen receptor

rect folding of the polypeptide chain; as a consequence, much of the mutated protein is not secreted but is retained in the ER and rapidly degraded even though, when properly folded, it could still function as ion channel at the cell surface ([35] and references therein).

Many proteins folds, one amyloid core structure

It is now well established that the molecular basis of protein aggregation into amyloid structures involves the existence of 'misfolded' forms of proteins, i.e. proteins that are not in the structures in which they normally function in vivo or of fragments of proteins resulting from degradation processes that are inherently unable to fold [4, 7, 8, 36]. Aggregation is one of the common consequences of a polypeptide chain failing to reach or maintain its functional three-dimensional structure. Such events can be associated with specific mutations, misprocessing phenomena, aberrant interactions with metal ions, changes in environmental conditions, such as pH or temperature, or chemical modification (oxidation, proteolysis). Perturbations in the conformational properties of the polypeptide chain resulting from such phenomena may affect equilibrium 1 in Fig. 1 increasing the population of partially unfolded, or misfolded, species that are much more aggregation-prone than the native state. Increased levels of aggregation-prone species may also occur as a

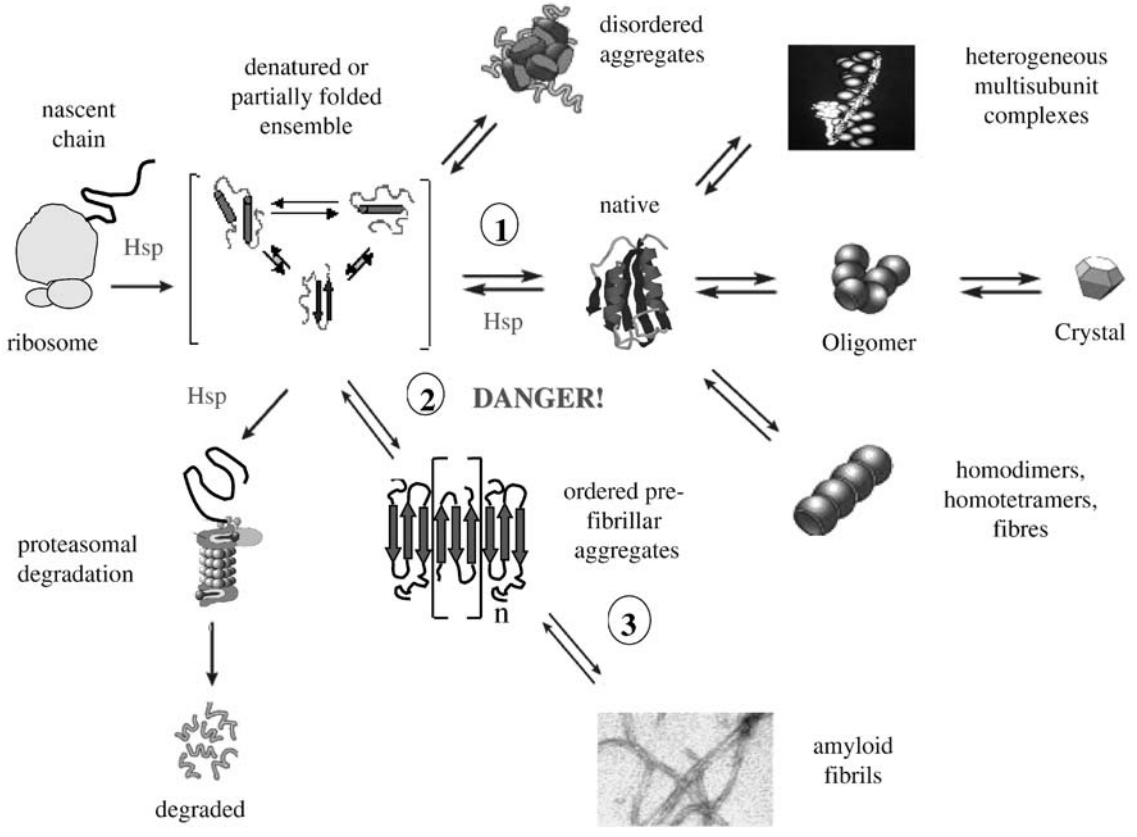

Fig. 1 Overview of the possible fates of a newly synthesised polypeptide chain. The equilibrium ① between the partially folded molecules and the natively folded ones is usually strongly in favour of the latter except as a result of specific mutations, chemical modifications or partially destabilising solution conditions. The increased equilibrium populations of molecules in the partially or completely unfolded ensemble of structures are usually degraded by the proteasome; when this clearance mechanism is impaired, such species often form disordered aggregates or shift equilibrium ② towards the nucleation of pre-fibrillar assemblies that eventually grow into mature fibrils (equilibrium ③). *DANGER!* indicates that pre-fibrillar aggregates in most cases display much higher toxicity than mature fibrils. Heat shock proteins (*Hsp*) can suppress the appearance of pre-fibrillar assemblies by minimising the population of the partially folded molecules by assisting in the correct folding of the nascent chain and the unfolded protein response target incorrectly folded proteins for degradation

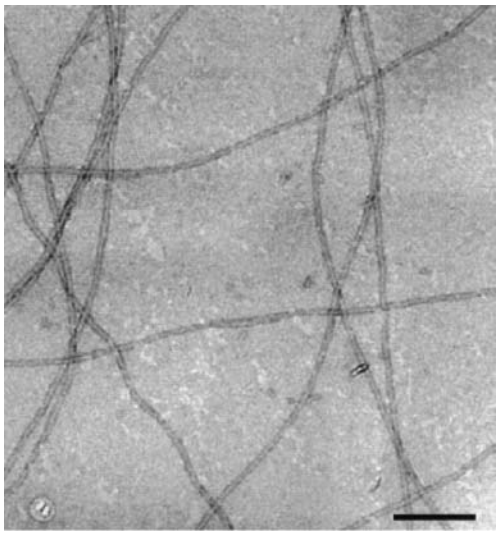

Fig. 2 Transmission electron microscopy of a mesh of amyloid fibrils assembled from human lysozyme negatively stained with uranyl acetate. *Scale bar*, 400 nm (From [42])

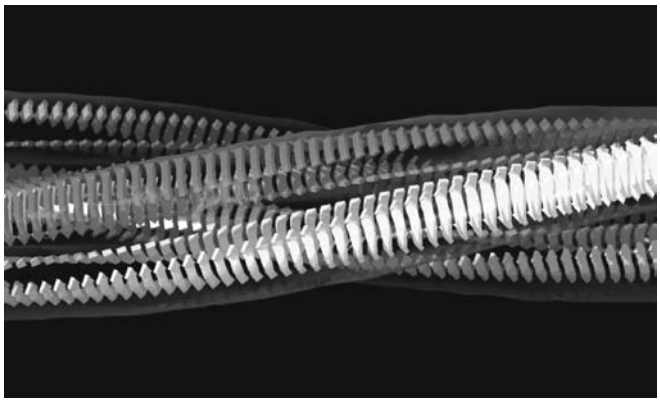

Fig. 3 Schematic drawing of the structural organisation of insulin fibrils. The image shows a fibril with four protofilaments wound around each other. In this model the core structure of each protofilament is a row of β-sheets (here running antiparallel) where each insulin molecule would occupy two layers connected by the interchain disulfide bonds (not shown) and each β-strand runs perpendicular to the fibril axis. The transparent surface depicts the density of the compact fibril under electron microscopy. (Reproduced with permission from [44])

consequence of enhanced protein synthesis or reduced clearance, particularly in the case of intrinsically unstable or natively unfolded proteins or proteins with relatively stable folding intermediates such as β_2-microglobulin [37]. Aggregation rate may also be increased by mutations affecting equilibrium 2 in Fig. 1 so as to kinetically favour aggregate nucleation. Reaction 3 is considered essentially, but not absolutely, irreversible, and mature fibrils are thought as the final, stable product of the aggregation process. Indeed, a number of findings support a regression of deposits and an arrest of new fibril formation following removal of the supply of fibril precursor proteins ([11] and references therein).

The various peptides and proteins associated with amyloid diseases have no obvious similarities in size, amino acid composition, sequence or structure. Nevertheless, the amyloid fibrils into which they convert have marked similarities both in their external morphology (Fig. 2) and in their internal structure (Fig. 3). Circular dichroism and Fourier transform infra-red spectroscopy both indicate a high content of β-structure, even when the monomeric peptide or protein is substantially disordered or rich in α-helical structure. Although it has not yet proved possible to obtain a detailed definition of the molecular structure of any amyloid fibril, investigations by electron and atomic force microscopy show that they are typically long, straight and unbranched. The fibrils are typically 6–12 nm in diameter and usually consist of two to six 'protofilaments', each of diameter about 2 nm, that are often twisted around each other to form supercoiled rope-like structures [38, 39]. Each protofilament in such structures appears to have a highly ordered inner core that X-ray fibre diffraction data suggest consists of some or all of the polypeptide chain arranged in a characteristic cross-β structure. In this structural organisation, the β-strands run perpendicular to the protofilament axis, resulting in a series of β-sheets that propagate along the direction of the fibril (Fig. 3).

Little is known at present about the detailed arrangement of the polypeptide chains themselves within amyloid fibrils, either those parts involved in the core β-strands or in regions that connect the various β-strands. Recent data suggest that the sheets are relatively untwisted and may in some cases at least exist in quite specific supersecondary structure motifs such as β-helices [6, 40] or the recently proposed μ-helix [41]. It seems possible that there may be significant differences in the way the strands are assembled depending on characteristics of the polypeptide chain involved [6, 42]. Factors including length, sequence (and in some cases the presence of disulphide bonds or post-translational modifications such as glycosylation) may be important in determining details of the structures. Several recent papers report structural models for amyloid fibrils containing different polypeptide chains, including the $A\beta_{40}$ peptide, insulin and fragments of the prion protein, based on data from such techniques as cryo-electron microscopy and solid-state magnetic resonance spectroscopy [43, 44]. These models have much in common and do indeed appear to reflect the fact that the structures of different fibrils are likely to be variations on a common theme [40]. It is also emerging that there may be some common and highly organised assemblies of amyloid protofilaments that are not simply extended threads or ribbons. It is clear, for example, that in some cases large closed loops can be formed [45, 46, 47], and there may be specific types of relatively small spherical or 'doughnut' shaped structures that can result in at least some circumstances (see below).

A very important aspect of the amyloid structures is the specific mechanism by which they are formed from

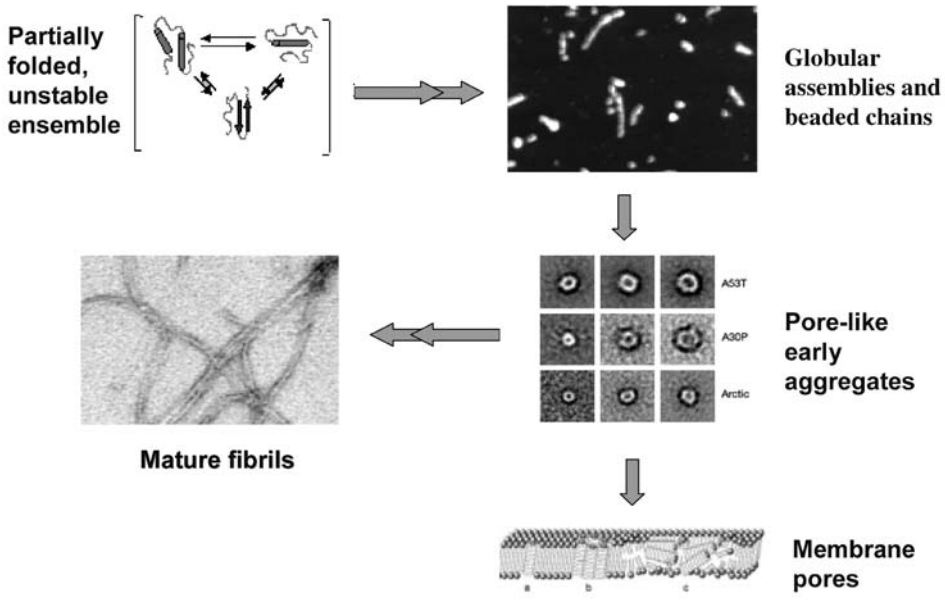

Fig. 4 Some amyloid-related peptides/proteins form early aggregates of globular appearance that further organise into beaded chains, globular annular 'doughnut' shaped assemblies eventually giving mature protofilaments and fibrils. Pre-fibrilar aggregates may interact with reconstituted phospholipid membranes and with cell membranes where they form aspecific channels (pores) disrupting cellular homeostasis. The latter possible mechanism of toxicity is similar to that displayed by antimicrobial peptides, pore-forming eukaryotic proteins and bacterial toxins and newly synthesised cyclic peptide antibiotics (see text). The electron micrographs of the globular and beaded chains of Aβ peptides are taken from Harper et al. [200]. The electron micrographs of the rings of the α-synuclein A53T (*upper row*) and A30P (*middle row*) mutants and of the Alzheimer precursor protein artic mutant (*lower row*) are from [201]

their soluble precursors. It is clear from studies of a variety of different systems including disease-related and disease-unrelated proteins that several more-or-less well-defined steps are involved in the assembly process [45, 46, 47, 48, 49, 50]. In most studies small particles can be seen, for example, in electron micrographs, which are often called 'amorphous aggregates' or sometimes 'micelles'. Structures known as protofibrils have frequently been observed, having a high β-sheet content but existing in heterogeneous populations of small, roughly spherical or tubular assemblies, 2.5–5.0 nm in diameter [46, 47, 49]. These species are often associated into bead-like chains or annular rings such as the 'doughnut' shaped species mentioned above and described further below (Fig. 4). Such assemblies appear, at least in most cases, to be precursors of longer protofilaments and mature fibrils that appear only after longer periods of time. As we discuss below, these 'early aggregates' may be very important for understanding the nature and origins of the pathological properties of amyloid structures associated with disease, and particularly with neurodegenerative conditions. A very recent report provides evidence that the soluble pre-fibrillar aggregates of differing peptides and proteins are equally recognised by polyclonal antibodies raised against pre-fibrillar assemblies made from $A\beta_{1-40}$ or $A\beta_{1-42}$ peptides, whereas the same antibodies are unable to recognise the corresponding fibrillar aggregates [51]. This finding is of value, indicating that pre-fibrillar aggregates of proteins and peptides as different as Aβ peptides, lysozyme, insulin, amylin, α-synuclein, the 106–126 prion peptide and poly(Q) stretches share common structural features recognised by the same antibody which are different from those displayed by the monomer/oligomer or fibrillar counterparts.

The similarity of some early amyloid aggregates with the pores resulting from oligomerisation of bacterial toxins and pore-forming eukaryotic proteins (see below) also suggest that the basic mechanism of protein aggregation into amyloid structures may not only be associated with diseases but in some cases could result in species with functional significance. Recent evidence indicates that a variety of micro-organisms may exploit the controlled aggregation of specific proteins (or their precursors) to generate functional structures. Examples include bacterial curli [52] and proteins of the interior fibre cells of mammalian ocular lenses, whose β-sheet arrays seem to be organised in an amyloid-like supramolecular order [53]. In this case the inherent stability of amyloid-like protein structure may contribute to the long-term structural integrity and transparency of the lens. Recently it has been hypothesised that amyloid-like aggregates of serum amyloid A found in secondary amyloidoses following chronic inflammatory diseases protect the host against bacterial infections by inducing lysis of bacterial cells [54]. One particularly interesting example is a 'misfolded' form of the milk protein α-lactalbumin that is formed at low pH and trapped by the presence of specific lipid molecules [55]. This form of the protein has been reported to trigger apoptosis selectively in tumour cells providing evidence for its importance in protecting infants from certain types of cancer [55]. This complex is presently in clinical trials as a therapeutic agent in the

treatment of various types of tumours and shows in a dramatic way that different conformational states of a protein can have very different biological properties.

Amyloid formation is a generic property of polypeptide chains

Until about 30 years ago proteolysis was considered to be the primary factor triggering the formation of amyloid aggregates in vivo, following the demonstration that lysosomal enzymes, at acidic pH values, are able to convert amyloidogenic proteins into amyloid fibrils [56]. This idea was challenged around 10 years ago when it was shown that transthyretin can be converted in vitro into amyloid fibrils following an acid-induced conformational change [57]. This finding demonstrated that a modification of the three-dimensional structure was sufficient to enable the production of an aggregation-prone species. This suggestion was not immediately accepted, at least in part as a consequence of the well established fact that the peptide found in the plaques characteristic of Alzheimer's disease resulted from proteolysis of the Alzheimer's precursor protein. Following these initial observations a large number of proteins known to aggregate in vivo were found to form fibrillar aggregates in vitro as a result of induced conformational changes; these data, however, reinforced the idea that the molecular basis of protein aggregation was an unusual feature of the few peptides and proteins found to be associated with the amyloid diseases, resulting from a specific conformational change related to the specific amino acid sequences. In 1998 two papers were published, each reporting the observation that a protein unrelated to any amyloid disease aggregated in vitro to form structures indistinguishable from the amyloid fibrils that could be produced from the disease-associated peptides and proteins [58, 59]. These observations were made by chance, but it was soon shown that a similar conversion could be achieved deliberately for other proteins by a rational choice of solution conditions [60, 61]. Since then a substantial number of similar studies have been reported ([61] and references therein; Table 3). In each case aggregation of a full-length protein to form amyloid fibrils was found to require solution conditions (such as low pH, lack of specific ligands, high temperature, moderate concentrations of salts or co-solvents such as trifluoroethanol) such that the native structure was partially or completely disrupted but under which interactions such as hydrogen-bonding were not completely inhibited.

It was also found that many peptides, both fragments of natural proteins or artificially designed sequences, that were unable to fold to stable globular structures readily formed fibrils [62]. Remarkably, it has become evident recently that peptides with as few as 4–6 residues can often form very well defined fibrils with all the characteristics of the amyloid fibrils formed by proteins often with 100 residues or more [62, 63]. These results provided strong support for the suggestion that the ability to form amyloid fibrils is a generic property of peptides and proteins, and that the structures were the result of the inherent physico-chemical properties of the polypeptide main-chain rather than the specific interactions of side-chains. The common structural features of amyloid fibrils can readily be explained as a simple consequence of the fact that the main chain is common to all sequences [64]. This finding contrasts with the large number of different folds characteristic of native proteins as in this case the structures are dictated primarily by interactions involving the side chains that differ for each protein sequence. That is not to say that the properties of the side chains are not important at all in amyloid structures, but simply that they do not define the core structure. It is clear that the presence of different side chains can influence the details of amyloid structures, particularly the assembly of protofibrils, and that they give rise to the variations on the common structural theme discussed above. More fundamentally, the composition and sequence of a peptide or protein affects profoundly its propensity to form amyloid structures under given conditions (see below).

Because the formation of stable protein aggregates of amyloid type does not normally occur in vivo under physiological conditions, it is likely that the proteins encoded in the genomes of living organisms are endowed with structural adaptations that mitigate against aggregation under these conditions. A recent survey involving a large number of structures of β-proteins highlights several strategies through which natural proteins avoid intermolecular association of β-strands in their native states [65]. Other surveys of protein databases indicate that nature disfavours sequences of alternating polar and nonpolar residues, as well as clusters of several consecutive hydrophobic residues, both of which enhance the tendency of a protein to aggregate prior to becoming completely folded [66, 67]. Very recent studies have examined

Table 3 Proteins unrelated to disease that form amyloid fibrils in vitro

Domain/protein	Year
SH3 domain p85 phosphatidyl inositol-3-kinase (bovine)	1998
Fibronectin type III module (murine)	1998
Acylphosphatase (equine)	1999
Monellin (*Dioscoreophyllum camminsii*)	1999
Phosphoglycerate kinase (yeast)	2000
B1 domain of IgG binding protein (*Staphylococcus*)	2000
Apolipoprotein CII (human)	2000
ADA2H (human)	2000
Met aminopeptidase (*Pyrococcus furiosus*)	2000
Apocytochrome *c* (*Hydrogenobacter thermophilus*)	2001
HypF N-terminal domain (*Escherichia coli*)	2001
Apomyoglobin (equine)	2001
Amphoterin (human)	2001
Curlin CgsA subunit (*Escherichia coli*)	2002
Vl domain (murine)	2002
Fibroblast growth factor (*Notophthalmus viridescens*)	2002
Stefin B (human)	2002
Endostatin (human)	2003

more general features of proteins that are likely to be important determinants of aggregation. In particular, studies have been carried out that explore the process of aggregation using approaches that have been used previously to probe the mechanism of protein folding and stability [68, 69]. These studies have revealed the importance of general physicochemical characteristics of polypeptide chains, such as the hydrophobicity and secondary structure propensities of specific regions of the sequence, and their overall charge [70, 71] (F. Chiti, M. Stefani, C.M. Dobson, Nature, in press). These findings indicate that the tendency of a polypeptide chain to aggregate rather than fold in vitro can be substantially perturbed by such factors as an enhanced β-strand propensity and hydrophobicity and a decreased overall charge.

The demonstration that peptides and proteins that are unrelated to disease have a generic ability to form amyloid fibrils has profound consequences for understanding the fundamental origins of the deposition of proteins in diseases as we discuss below. It also has very great practical relevance as it increases dramatically the number of proteins and peptides that one can investigate in order to discover the general features underlying the mechanisms of protein misfolding and aggregation. Moreover, it has prompted a more general discussion of the various states that could be adopted by polypeptides following their synthesis in vivo and of the way that biology has exploited to regulate these through evolutionary processes. Thus, for example, as we discuss below, the translocation of proteins across membranes is likely to involve partially folded states similar to those that are populated during the folding process through which the native state is achieved. Moreover, the evolution of the highly cooperative nature of native protein structures appears to be a critical step in permitting proteins to avoid aggregation for significant lengths of time [72]. It is clear, however, that the functional state of a polypeptide chain is in dynamic equilibrium with other states including aggregates and their precursors (Fig. 1). Therefore the formation of aggregates, and in particular the build-up of the concentrations of the precursor species that nucleate rapid aggregate formation, can be triggered by changes in conditions.

One crucial factor in this regard is simply the concentration of aggregation-prone species present under different circumstances. It is clear that enhanced expression levels of certain proteins and increased concentrations of incompletely folded states of globular proteins as a result of mutagenesis are factors associated with some forms of amyloidosis. Other factors of importance more generally could be the perturbation of the environment where aggregation-prone species are present. One interesting example of this phenomenon concerns the change in the total concentration of molecules within a given biological compartment. Indeed, the phenomenon of 'molecular crowding', a result of the very high overall concentration of macromolecules in cells (typically 300–400 mg/ml) is known to perturb the folding and binding of proteins [73]. In this context it has been estimated that an increase in macromolecular crowding from 30% to 33% (the figures represent the volume of a given space occupied by molecules) can cause a rise by as much as an order of magnitude in molecular binding affinities [74]. Such a change, which could, for example, be associated with alterations in cellular properties as a result of ageing [75] or of progression through the cell cycle [76], would favour more compact states of macromolecules, and also increase the population of aggregated species including those that nucleate rapid growth of amyloid fibrils. In this regard it has been shown that every type of cell is equipped with mechanisms designed to maintain or to restore cellular volume, water content, and/or turgor pressure in response to any changes in the composition of the extracellular fluid [77].

Precursors of amyloid fibrils can be toxic to cells

It was generally assumed until recently that the proteinaceous aggregates most toxic to cells are likely to be mature amyloid fibrils, the form of aggregates that have been commonly detected in pathological deposits. It therefore appeared probable that the pathogenic features underlying amyloid diseases are a consequence of the interaction with cells of extracellular deposits of aggregated material. As well as forming the basis for understanding the fundamental causes of these diseases, this scenario stimulated the exploration of therapeutic approaches to amyloidoses that focused mainly on the search for molecules able to impair the growth and deposition of fibrillar forms of aggregated proteins. An increasing quantity of recent experimental data suggests, however, that in many cases at least the species that are most highly toxic to cells are the pre-fibrillar aggregates (sometimes referred to as amorphous aggregates, protein micelles or protofibrils) rather than the mature fibrils into which they often develop. In particular, a number of reports concerning Aβ peptides, α synuclein and transthyretin indicate that these early aggregates are the most toxic species [18, 76, 77, 78, 79, 80]; in addition, the presence of such species has also been reported for huntingtin [44], and possibly the androgen receptor [81] in diseased transgenic mice. The hypothesis that toxicity is exhibited primarily by early aggregates also provides an explanation for the lack of existence of a direct correlation between the density of fibrillar plaques in the brains of victims of Alzheimer's disease and the severity of the clinical symptoms [82].

These data are particularly interesting in the light of recent investigations of the effects on cells of aggregates of two proteins that are not associated with disease, the src-homology 3 (SH3) domain of the phosphatidyl inositol-3-kinase and the N-terminal domain of the bacterial hydrogenase maturation factor HypF (HypF-N). These small proteins had already been found to convert into amyloid fibrils in vitro under carefully chosen conditions [58, 61], and it was found that the process of formation of mature fibrils can be manipulated such that it involved

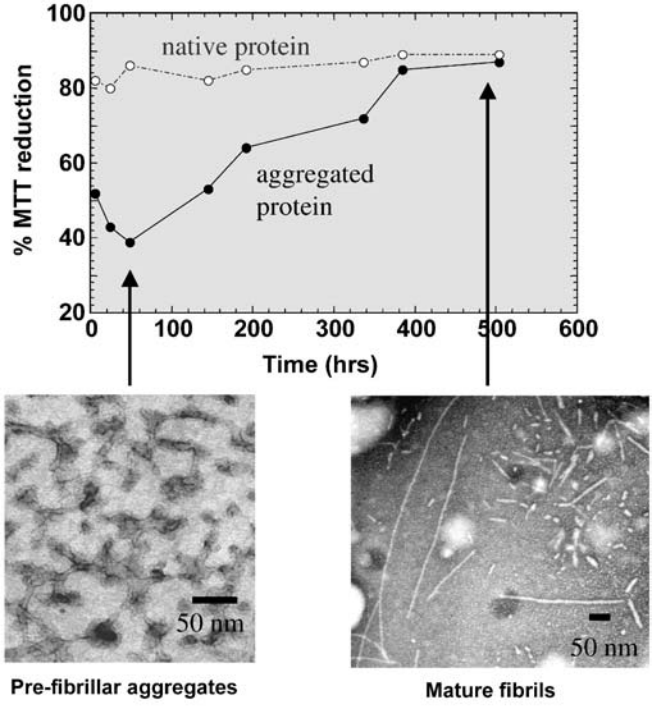

Fig. 5 Amyloid aggregates of the N-terminal domain of the bacterial hydrogenase maturation factor HypF, a protein unrelated to any amyloid disease, are cytotoxic in their pre-fibrillar organisation. Protein samples at differing times and stages of aggregation were added to the culture medium of NIH-3T3 or PC12 cells. Only globular, pre-fibrillar aggregates displayed cytotoxicity, whereas mature fibrils were substantially harmless. (Modified from [50])

a series of relatively well defined pre-fibrillar species. These pre-fibrillar aggregates, formed early in the aggregation process of both proteins, were found to impair substantially the viability of cultured cells when added to the culture medium. By contrast, the mature fibrils were essentially harmless (Fig. 5) [50]. These findings are remarkable as they show that the aggregates produced in vitro by proteins unrelated to disease are not only effectively identical to those formed by disease-related polypeptides, but that the pre-fibrillar species can also be as highly cytotoxic [50]. This result implies that amyloid cytotoxicity may arise from common characteristics of the supramolecular structure of the aggregates rather than from any specific features of the amino acid sequences of the monomer polypeptides. Both of these features are in direct contrast to the properties of native proteins, where the sequences determine completely both the native structure and its biological effects. Such an otherwise remarkable result in fact follows from the conclusion discussed above that the specific interactions of the side chains need not to determine directly the structure and hence the properties of species other than the native state.

Although further studies of a wider range of peptides and proteins are required to explore the generality of these observations, it may well be that the cytotoxicity of these types of aggregate is generic [50]. Such toxicity is likely to arise from the 'misfolded' nature of the aggregated species and their precursors and from the exposure in such species of regions of the protein (e.g. hydrophobic residues and the polypeptide main chain) that are buried in the native state. As many of these regions are likely to be aggregation-prone (or 'sticky') they may be able to interact with membranes and other cellular components [50]. Indeed, in an ensemble of small aggregates containing misfolded polypeptide chains there will be a vast array of exposed groups of amino acids, some of which may mimic regions of the surfaces of native proteins. Such aggregates are therefore likely to be able to interact inappropriately with the binding partners or receptors of a wider range of different proteins. Indeed, the intrinsic instability of pre-fibrillar species that enables them to assemble further and become organised into more highly ordered structures itself reflects the existence of accessible regions of the structures. In accord with these conclusions, pre-fibrillar assemblies have been shown to interact with synthetic phospholipid bilayers [45, 48, 83, 84, 85, 86, 87] and with cell membranes [45, 54, 88], possibly destabilising them and impairing the function of specific membrane-bound proteins [89, 90]. Pre-fibrillar amyloid aggregates may interact with cell membranes in a way that is reminiscent of the action of a number of prokaryotic or eukaryotic peptides or proteins (e.g. some bacterial toxins) that oligomerise into the membranes of the target cells forming pore-like assemblies that destabilise cell membranes and impair ion balance across these structures (see below).

The hypothesis that proteins and peptides able to destabilise cell membranes are intrinsically cytotoxic depicts membrane damage as a common molecular basis for malfunctioning and impairment of viability in cells exposed to cytotoxic peptides such as those present in venoms, antimicrobial secretions, the mammalian immune system, antibacterial toxins, viral proteins, misfolded proteins or amyloid aggregates [90, 91]. This scenario is supported by the findings mentioned above concerning the toxic effects on tumoural cells of a partially unfolded state of α-lactalbumin at acidic pH [55], and on the proposed mechanism of membrane insertion and toxicity of perfringolysin, whose insertion into the plasma membrane of the target cells requires a conformational change resulting from the conversion of the six α-helices in domain 3 into four amphipathic β-strands [92]. Interestingly, 15 years ago it was suggested that the 'molten globule' states of proteins are important in enabling proteins to cross cell membranes [93]. This idea has now been supported by the finding that pre-fibrillar aggregates, whose exposed hydrophobic surfaces have similarities to those of molten globules, are able to interact and associate with cell membranes.

It is possible that in some cases misfolded proteins and their aggregates generate toxicity and impairment of cellular function by mechanisms different from those outlined above, as it could be the case of the aggregates of tau protein and proteins containing poly(Q) exten-

sions; however, it is the delineation of such a mechanism that represents an important step on the way to a generalised description of the pathological effects of amyloid formation. The structural data obtained by the study of bacterial toxins (see below) provide a specific model of a possible mechanism of interaction of pre-fibrillar amyloid assemblies with cell membranes leading to cell death [45, 46, 48]. It can also give clues as to the nature of conformational modifications underlying protein oligomerisation into β-sheet-rich structures such as pore-forming toxins and pre-fibrillar aggregates. It also has fascinating consequences, for example, suggesting that bacteria could have evolved specific toxins to replicate in a controlled and efficient manner the less optimised effects of aggregates that are precursors to mature amyloid fibrils. At this point it is interesting to note that the hypothesis that pre-fibrillar assemblies display high toxicity has important consequences with respect to the design of any therapeutic approach aimed at the treatment or prevention of amyloid disease. It is important that if therapeutic intervention is targetted at inhibition of fibril growth, it should not increase the population of pre-fibrillar species. There are many reasons to suppose that the latter situation can be avoided, but it makes particularly attractive any therapeutic strategy aimed at reducing the presence in cells of aggregation-prone monomers rather than interrupting their aggregation into fibrillar species. This point has recently been emphasised through studies of a mouse model of Huntington's disease [94]. Indeed, it may well be that the low toxicity of mature fibrils relative to their precursors represents an important protective mechanism for biological systems. Such a mechanism is, however, likely to be effective only at the stage of a disease where rapid aggregation has already been initiated.

Structural basis and molecular features of amyloid toxicity

The presence of toxic aggregates inside or outside cells can impair a number of cell functions that ultimately lead to cell death by an apoptotic mechanism [95, 96]. Recent research suggests, however, that in most cases initial perturbations to fundamental cellular processes underlie the impairment of cell function induced by aggregates of disease-associated polypeptides. Many pieces of data point to a central role of modifications to the intracellular redox status and free Ca^{2+} levels in cells exposed to toxic aggregates [45, 89, 97, 98, 99, 100, 101]. A modification of the intracellular redox status in such cells is associated with a sharp increase in the quantity of reactive oxygen species (ROS) that is reminiscent of the oxidative burst by which leukocytes destroy invading foreign cells after phagocytosis. In addition, changes have been observed in reactive nitrogen species, lipid peroxidation, deregulation of NO metabolism [97], protein nitrosylation [102] and upregulation of heme oxygenase-1, a specific marker of oxidative stress [103]. Moreover, it has been shown that cells can be protected against aggregate toxicity by treatment with antioxidants such as tocopherol, lipoic acid and reduced glutathione [104]. In this regard an interesting study in prion-infected mice suggests that an increase in free radical production, accompanied by a reduction in the efficacy of mitochondrial anti-oxidant defences, is responsible for damage to the brain, and that this effect contributes generally to the development of the prion diseases [105]. Recent data also point to direct effects of ageing and oxidative stress on the activity and expression levels of the proteasome and hence on cell viability in nerve tissue [106]. Inhibition of the proteasome is likely to result in the accumulation of oxidised or otherwise damaged proteins thus increasing the detrimental effects of ROS damage. A possible role of Hsp27 in preventing polyglutamine cytotoxicity by suppressing ROS production has also recently been proposed in cells transiently transfected with vectors expressing exon 1 to produce varying lengths of the glutamine repeat sequence in huntingtin [107, 108]. These data suggest a possible way that oxidative stress following exposure to the early species involved in amyloid formation could damage cells and eventually cause cell death.

It is not clear why protein aggregation is followed, even in vitro, by production of ROS. In the case of $A\beta_{42}$, Met35, Gly29 and Gly33 have been suggested to be involved [109]; a role has also been proposed for metal ions such as Fe, Cu and Zn, for example, through the generation of hydroxide radicals from hydrogen peroxide [110, 111]. An upregulation of the activity of hydrogen peroxide-producing membrane enzymes, such as plasma membrane NADPH oxidase and ER cytochrome P450 reductase, has also been reported in Aβ-induced neurotoxicity in microglia and in cortical neurons [112, 113]. More generally, intracellular oxidative stress could be related to some form of destabilisation of cell membranes by toxic species leading to a failure to regulate appropriately plasma membrane proteins such as receptors and ion pumps [114] and/or to impairment of mitochondrial function. Mitochondria play a well recognised role in oxidative stress and apoptosis; in this regard, a key factor in Aβ peptide neurotoxicity could be the opening of mitochondrial permeability transition pores by Ca^{2+} entry in neuronal mitochondria [115] followed by release of cytochrome *c*, a strong inducer of apoptosis.

It has been suggested recently that intracellular ROS elevation following exposure to amyloid aggregates is a consequence of Ca^{2+} entry into cells followed by stimulation of oxidative metabolism aimed at providing the ATP needed to support the activity of membrane ion pumps involved in clearing excess Ca^{2+} [116]. ROS elevation would in turn oxidise not only the proteins involved in ion transfer but also proteins such as calmodulin [116] that when oxidised is unable to activate the Ca^{2+}-ATPase. The down-regulation of the Ca^{2+}-ATPase activity would then reduce the need for ATP, and hence ATP synthesis and ROS production by oxidative metabolism, leading to an increase in intracellular Ca^{2+} concen-

tration [116]. The same could happen in old age, where the ATP levels tend to be lower resulting in an energy deficiency. This hypothesis can explain the relationship between ROS, apoptosis, mitochondrial damage and intracellular free Ca^{2+} increase shown by cells exposed to toxic amyloid aggregates [91, 108, 117, 118]. Indeed, many studies have suggested a close relationship between Alzheimer's, Parkinson's and prion diseases and the dysregulation of calcium homeostasis. As pointed out above, the increase in intracellular free Ca^{2+} levels is thought to be a consequence of the impairment of membrane permeability; the latter may be a consequence of the presence into the membrane of aspecific amyloid pores or may follow oxidative stress, membrane lipid peroxidation producing reactive alkenals such as 4-hydroxynonenal, and the chemical modification of membrane proteins acting as ion pumps [98, 119]. Very recent data have shown that cells exposed to early aggregates of a protein unrelated to disease (HypF-N) display increased ROS and enhanced free Ca^{2+} levels (M. Bucciantini, C.M. Dobson, M. Stefani, unpublished results). These findings suggest that the impairment of viability in cells in the presence of at least some of the aggregates of small proteins can be related to the disruption of the same biochemical processes found to be affected by similar aggregates of disease-related proteins and peptides. The latter idea is supported by recent findings indicating that antibodies raised against pre-fibrillar aggregates of Aβ peptides and able to recognise similar assemblies made by other proteins and peptides as discussed above can reduce the toxicity of these species to cultured cells [51]. This finding supports the idea of a common mechanism for the toxicity of protofibrils formed from structurally different peptides and proteins, which are independent of the structural features of the monomer form but are linked to common structural features displayed in their pre-fibrillar organisation [51].

The channel hypothesis of amyloid toxicity

As outlined above, pre-fibrillar amyloid aggregates may interact with cell membranes in a way that is reminiscent of the action of eukaryotic pore-forming proteins such as peptides found in venoms and antimicrobial secretions [90], bacterial toxins [92], perforin [120], the C5b-8/9 complement assembly in the membrane attack complex [121] and the BCL-2 family of pro-apoptotic and anti-apoptotic proteins ([122] and references therein). The ring-shaped dynamin oligomers and their stacked, twisted assemblies, thought to play a role in clathrin-coated vesicle fission [123] are also apparently similar to the 'doughnut' shaped rings formed by at least some pre-fibrillar amyloid aggregates; however, in some cases pores may be formed even under conditions where 'doughnuts' are not observed (Fig. 4). In most of these examples, such as those involving the eukaryotic pore-forming proteins, the molecular basis of protein oligomerisation into pore complexes has not been described in detail. Some pore-forming toxins are known to permeate and permeabilise plasma membranes of the host cells leading to disruption of intracellular homeostasis and eventually cell death. In general, these molecules form β-barrel oligomers where each monomer contributes one or two amphipathic β-hairpins to the formation of the transmembrane pore [92, 124, 125]. Such variously sized (1–2 to 20–30 nm), sodium dodecyl sulphate resistant, high β-sheet content complexes result as a consequence of a major conformational change, possibly via a partially unfolded intermediate or of a conversion of specific α-helices into amphipathic β-strands. In most cases, such structural modifications are triggered by specific proteolytic cleavage processes or by interaction with membranes favouring the assembly of pre-pore oligomeric assemblies competent for insertion into membranes [126].

Association of complement components into the C5b-8/9 complexes also requires partial unfolding and exposure of hydrophobic surfaces and leads to the formation of ring-shaped complexes that insert deeply into the membrane lipid bilayer and form small 'leaky' pores [121, 127]. Interestingly, recently developed antibacterial agents based on the cyclic D,L-α-peptide architecture are thought to function by forming β-sheet-like open-ended tubular structures in lipid membranes [128]. On such a model the antibacterial activity would also be consequence of an increase in membrane permeability. Indeed, a mechanism similar to those mentioned above has been suggested for amyloid aggregates from studies of the aggregation of $Aβ_{1–28}$ that show preferential binding of the peptide to acidic phospholipid domains [129]. The suggestion was made that binding could be followed by a structural transition from random to β-structure coupled to self-association within the membrane. The latter could then result in the local disruption of the membrane bilayer, allowing inappropriate ion trafficking between intra- and extra-cellular spaces. More recently it has been reported that in the presence of membranes low in cholesterol $Aβ_{1–40}$ is found at the membrane surface in a β-sheet structure, whereas its insertion into cholesterol-rich membranes favours a β-sheet to α-helix transition and reduces fibril formation [130]. A similar effect has been seen in a model peptide system in the presence of varying concentrations of lipid molecules; furthermore, recent findings indicate that incubation of spherical protofibrils of α-synuclein with brain-derived membranes produces membrane-bound pore-like annular protofibrils [88].

Since 1993, a 'channel hypothesis' of the molecular basis of the cytotoxicity of amyloid aggregates has been put forward [131] by similarity with the proposed mechanism of toxicity of pore-forming peptides and proteins [90, 91]. As is pointed out above, this idea stems from a number of pieces of evidence leading to the proposal that unchaperoned, positively charged and misfolded proteins, or early aggregates of such species, can interact with lipid membranes ([90, 91] and references therein). Evidence for this proposal comes from the study of both artificial model systems, such as phospholipid bilayers,

and cell membranes; in the latter the function of specific membrane proteins has been found to be impaired [78, 91]. In most cases interaction of a misfolded species with a membrane would occur via a two-step mechanism involving electrostatic interaction of the positively charged residues with negatively charged or polar lipid head groups followed by the insertion of hydrophobic regions into the membrane hydrophobic interior [91].

According to this hypothesis, misfolding of proteins, such as at least some of those involved in neurodegenerative diseases, would then induce cytotoxicity. Such cytotoxicity would be a direct consequence of the exposure of hydrophobic regions, favouring the interaction of the misfolded species with the plasma membrane and other cell membranes and leading to membrane damage via the formation of non-specific ion channels. These channels, or pores, have been described for a number of peptide and proteins associated with amyloid disease including Aβ peptides [19, 45, 78, 89] and their fragments [132], α-synuclein [133], islet-amyloid polypeptide [86], the 106–126 fragment of the prion protein [41], poly(Q) stretches [134, 135], the C-type natriuretic peptide [84], β_2-microglobulin [48], transthyretin [136], murine serum amyloid A [137] and the N-terminal peptide of an acute-phase isoform variant of human serum amyloid A1.1 (SAAp) [54]. The channels have been investigated primarily by recording ion currents across biological or reconstituted membranes, but 'doughnuts' of channel-like assemblies of pre-fibrillar aggregates of $A\beta_{1-42}$, α-synuclein, transthyretin and serum amyloid A have also been observed by electron and atomic force microscopy [45, 46, 49, 137].

In general, heterogeneity of amyloid intermediates, including globules, chains, doughnuts, protofilaments and fibrils, could result in increased potency of toxicity since the different types of intermediates may act in differing ways on membranes such as the suite of peptides in venoms. In the case of α-synuclein the 'pores' coexist with fibrils under conditions of molecular crowding [138], raising the possibility that the former are more stable under cytoplasmic conditions and leading to the proposal that they are the pathogenic species in Parkinson's disease [46]. The size-dependent permeabilisation of artificial vesicles by protofibrillar α-synuclein suggests that permeabilisation occurs mainly as a result of a specific membrane perturbation via the formation of pores at least 2.5 nm in diameter [46]. If α-synuclein annular protofibrils are the pathogenic species in Parkinson's disease and other amyloidoses, inhibition of their production should represent a suitable therapeutic strategy. However, it is difficult to imagine a drug molecule able to distinguish specifically among chain protofibrils, annular protofibrils and mature fibrils, when one considers that protofibril elongation into fibrils and protofibril annulation are likely to involve the same interactions leading to β-sheet extension [88]. The involvement of pores in the onset of the pathogenic cascade in vivo raises the need to develop small molecules able to inhibit membrane permeabilisation in vitro and suitable to be tried in animal models of Parkinson's and other diseases [88].

Overall, when considered together with the findings on pore-forming proteins, amyloid toxicity through channel formation could be featured as an undesired side effect of a mechanism of cell death conserved from bacteria to mammals.

The channel hypothesis does not necessarily diminish the importance of oxidative stress as a key perturbation to cells exposed to toxic amyloid aggregates. Instead it suggests that the initiation of the events that result in apoptosis is the entry of extracellular Ca^{2+} into cells and its accumulation in mitochondria. This process is then followed by the opening of pores that results in mitochondrial permeability and the release of cytochrome *c*. The entire process could be amplified by oxidative stress as the latter causes impairment of the membrane ion pumps. In addition, oxidative stress may itself be cytotoxic as it results in damage to proteins, thus leading to the condition known as 'chaperone overload' that is discussed below and by triggering intracellular apoptotic signals such as stress-activated protein kinases. The latter has been reported to occur in Alzheimer's disease [96, 139, 140]. Figure 6 summarises the proposed molecular events that are thought to lead to the death of cells following exposure to these toxic aggregates. It can be seen in this proposal that a key role in preventing damage to cells is played by the cellular clearance mechanisms. Indeed, impairment of these protective devices increasingly appears to be a central feature of the events leading to pathogenesis, as we discuss further below.

Although the idea of a single common molecular mechanism for the toxicity induced by specific pre-fibrillar forms of amyloid aggregates is particularly fascinating, the data presently available do not exclude other mechanisms of toxicity that are independent of, or additional to, the direct consequences of membrane destabilisation (e.g. by impairment of ion pumps), pore formation, and/or increases in ROS and free Ca^{2+} levels. Results have recently been reported concerning the toxicity towards cultured cells of aggregates of poly(Q) peptides which argues against a disease mechanism based on specific toxic features of the aggregates. These results indicate that there is a close relationship between the toxicity of proteins with poly(Q) extensions and their nuclear localisation. In addition they support the hypotheses that the toxicity of poly(Q) aggregates can be a consequence of altered interactions with nuclear coactivator or corepressor molecules including p53, CBP, Sp1 and TAF130 or of the interaction with transcription factors and nuclear coactivators, such as CBP, endowed with short poly(Q) stretches ([95] and references therein). They also show that pre-fibrillar aggregates added to the culture medium cross the plasma membrane and enter the cytoplasm. This observation is particularly interesting as it would support the idea that extracellular protein aggregates present in tissue can re-enter cells and thereby give rise to greater toxicity [141].

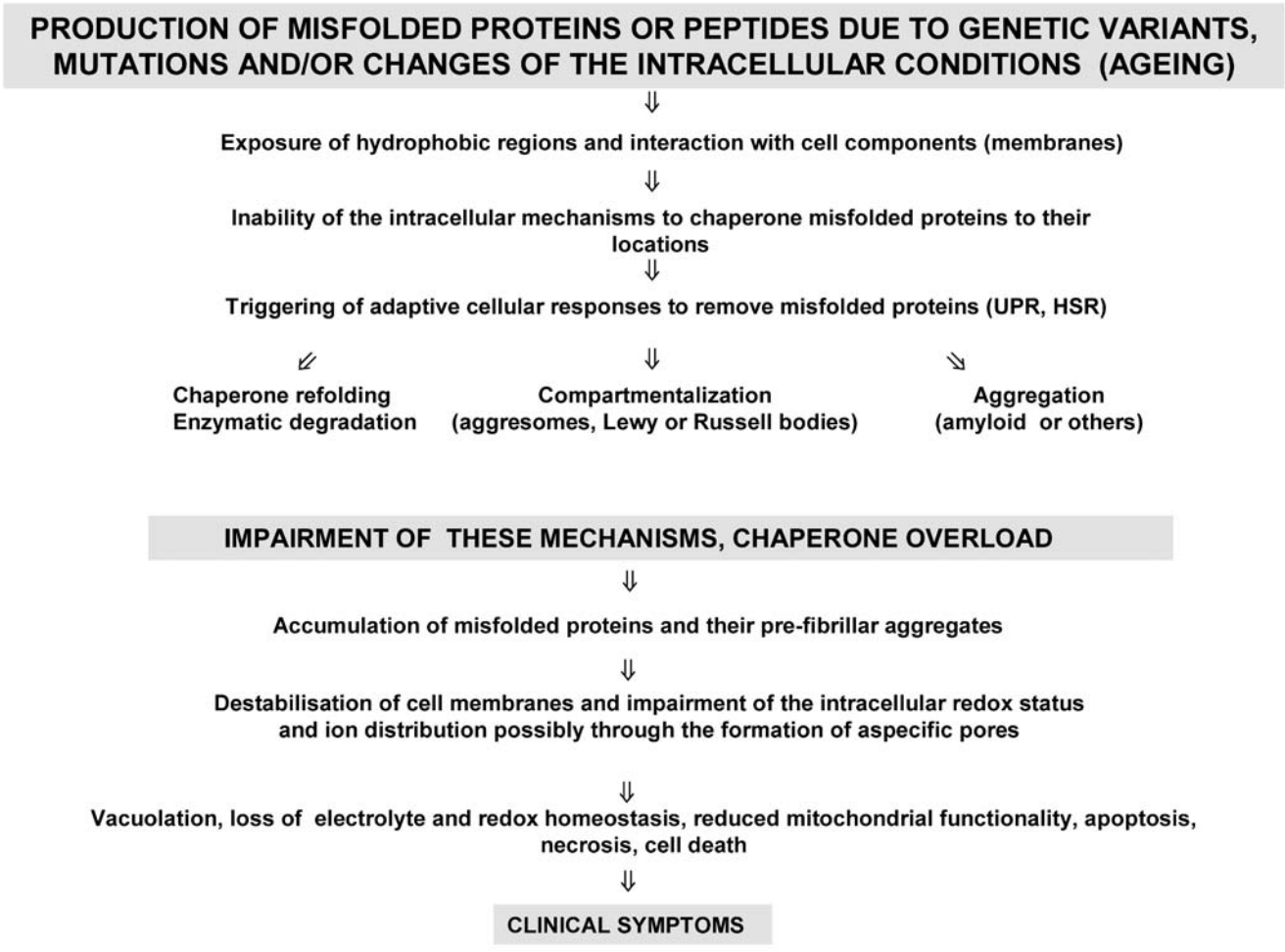

Fig. 6 Flow-chart of the main molecular steps leading misfolded polypeptide chains to induce cell death. In the panel, aggregation of proteins into fully formed, mature amyloid fibrils could be considered to be potentially beneficial in the light of recent findings indicating that, at least in most cases, the true toxic species are the early pre-fibrillar aggregates, whereas mature fibrils appear less toxic or devoid of toxicity. Degradation of misfolded proteins is carried out by the ubiquitin-proteasome machinery. The path leading to cell death occurs when the chaperone and clearing cellular machineries are overwhelmed by the presence of an excess of unfolded/malfolded proteins; the latter is followed by the appearance of unstable amyloid nuclei and pre-fibrillar assemblies further growing into mature fibrils; such assemblies may also interact with cell membranes destabilising them and modifying ion balance possibly by formation of aspecific membrane pores. The rise of the intracellular free Ca^{2+} and ROS is one of the earliest modification in the path of cell death following cell exposure to early amyloid aggregates of most peptides and proteins. (Modified from [91])

Consequences of the generic toxicity of protein aggregates

The recent data on the generic nature and properties of amyloid fibrils and their precursors suggest the need for a 'new view' of the molecular basis of amyloid toxicity. Instead of being the consequences of the interaction of cells with fibrils from the very limited number of proteins or peptides found in specific degenerative diseases, aggregate cytotoxicity appears to be a reflection of a more general property of misfolded proteins. As discussed above, a probable origin, at least in part, of this phenomenon is that misfolded proteins, and small aggregates of such species, have exposed hydrophobic regions such that they can interact with cell membranes, modifying their structural integrity and impairing their functional properties. According to this scenario, the toxic 'gain of function' of the aggregates may be due at least in part to their ability to destabilise and permeabilize cell membranes resulting in modifications of the intracellular environment that target the cells towards apoptotic or necrotic death. The recent reports emphasising the existence of a direct relationship between intracellular Ca^{2+} increases and ROS levels (see above) provide support for this idea, although exceptions may exist [95, 141]. Moreover, the hypothesis that a much larger number of proteins than previously suspected may give rise to aggregates that are cytotoxic raises the possibility that even minute deposits of early aggregates of as yet unidentified peptides or proteins accumulate within cells as a consequence of the ageing process. These aggregates could result from impairment of specific cellular functions, such as the ability to clear misfolded proteins or protein aggregates, or from changes in the intracellular environ-

ment such as a decrease in ambient pH or in the antioxidant defences or an increase in the macromolecular crowding (see above). The presence in cells of minute amounts of early aggregates could perhaps account for subtle impairments of cellular function and viability in the absence of a clear amyloid phenotype in systemic and neurological disorders that presently are not associated with amyloid deposition [50] as it can also be the case of prion disease elicited by certain prion protein variants [142].

This general hypothesis is made more intriguing in the light of the recent data concerning the variability of the human genome sequence, whose exons contain about 60,000 single nucleotide polymorphisms (an average of one or two per gene) [143]. At least some of these variations are likely to impair the ability of the proteins involved to fold efficiently to the stable native structure. Indeed, at 37°C the value of $\Delta G_{U \rightarrow F}$ for a typical protein is in the range of 5–15 kcal/mol. Destabilisation of the native structure by as little as 2 kcal/mol (that can easily occur following mutation or chemical modification, such as oxidation or altered glycosylation patterns) would, on a simple two-state model, enhance the equilibrium concentration of unfolded molecules by a factor of more than 30. It has been estimated that such a change would increase the probability of the nucleation of aggregation by a factor of more than 10^5 [144]. The possibility that the risk of acquiring Parkinson's disease is influenced by genetic polymorphisms is presently under investigation [145]. Another possibility is that it is linked to epigenetics (the complex changes in the genome, such as DNA methylation or histone acetylation, that do not affect the DNA sequence but modulate gene expression). It is increasingly believed that this phenomenon plays a highly important role in the development of common diseases, and its importance in cancer is well established [146]. Epigenetics could be part of the explanation of why most amyloid diseases appear late in our lifespan, as ageing is accompanied by changes in DNA methylation; at least in some cases this could lead to upregulation of the expression of specific proteins, stimulating their accumulation and aggregation into cells.

Taken together, such ideas suggest that toxic aggregates could in principle accumulate in vivo from a wide range of proteins under at least some conditions. However, this normally does not happen in normally functioning organisms due to the extraordinarily effective quality control mechanisms such as the unfolded protein response and the Hsps. In some cases negative selection against proteins with a high tendency to aggregate must occur in a context where folding-defective proteins are being produced rapidly as in the case of the Ig production by lymphocytes. Indeed, the occurrence of light-chain amyloidosis (see Table 1) as a consequence of enhanced Ig production is low, even in severe inflammatory conditions. This result implies the existence in plasma cells of a particularly efficient series of quality control mechanisms that enable selection against such mutants [147]. Any decrease in the efficiency of the cellular protection and clearance mechanisms would make the cell susceptible to damage and then targetted for apoptotic or necrotic death. Hence aggregate-induced cell death could often be a stochastic event rather than resulting from a slow accumulation in cells of defects arising from the presence of aggregates, as suggested in the recently proposed one-hit model of cell death in inherited neuronal degeneration [24]. This model suggests that the amyloidoses, and perhaps other degenerative conditions, are not just a consequence of the high propensity of some proteins to aggregate but are also associated with failures of the 'housekeeping' mechanisms that generally prevent the accumulation of misfolded and aggregated proteins in living systems.

The molecular consequences of biological evolution

The potential cytotoxicity of many aggregated proteins suggests that, in addition to providing cells with mechanisms to clear unfolded and misfolded proteins and to minimise their ability to induce toxicity, evolution must also have operated to eliminate protein sequences with a high intrinsic propensity to aggregate [8]. Thus mutations that are neutral with respect to protein function could be selected against because they enhance the tendency of proteins to aggregate under physiological conditions. It is interesting in this regard that most of the polypeptide chains associated with aggregation diseases are either intact, or fragments of, proteins that are secreted or membrane bound. It could be that such proteins are more easily able to escape the cellular mechanisms that protect against misfolding and aggregation. Moreover, it is possible that processing in the ER prior to secretion through the Golgi, or indeed the events involved in the retrograde translocation into the cytosol of polypeptide chains that have failed the quality-control tests in the ER [35], represent additional steps associated with folding in which errors could occur or accumulate. The recent studies of the ways in which structural adaptations of proteins can minimise their tendency to misfold and aggregate mentioned above show, however, that polypeptides are far from optimised in their ability to resist aggregation. One reason for this fact is that sequences must encode many features of proteins, such as their need to fold and to bind to other species. Another is that sequences selected by evolution are in general optimised only to an extent that allows a particular organism to function efficiently during its normal life span [148].

Conclusions of the type discussed above are also generally consistent with the known sequence features of the 'natively unfolded proteins' [3, 149]. This term refers to members of a large family of apparently unrelated proteins that includes many transcription factors, ribosomal proteins and also signalling proteins involved in cell cycle control at the transcriptional and translational levels. These proteins appear to be partly or completely intrinsically disordered in vitro and probably in many cases even in the cellular environments, although many un-

doubtedly adopt specific three-dimensional structures upon interaction with specific target proteins [150]. Unstructured domains are also found in certain regions of other proteins that are otherwise natively folded. A recent search in the Swiss Protein Database, for example, has led to the prediction that over 15,000 proteins contain disordered regions of at least 40 consecutive residues. Indeed, over 1000 proteins were suggested to be essentially completely disordered [151]. This observation indicates that significant segments of the eukaryotic genomes encode long stretches of amino acid residues that under some conditions at least are likely to be unfolded or to adopt non-globular structures of unknown nature.

Natively unfolded proteins are usually easily recognizable from the amino acid content as they generally display a low mean hydrophobicity and a high net charge. These characteristics, thought to be the molecular basis by which these proteins remain unfolded in the absence of partners, are also able to reduce their intrinsic tendency to aggregate in the highly crowded intracellular milieu [73]. In addition, the unstructured state of these proteins is likely to favour their rapid degradation by the cellular clearance mechanisms, a characteristic commonly revealed by their rapid intracellular turnover [149]. The latter characteristic could be an advantage for certain cellular functions, providing a further level of control to enable the cell to respond rapidly and effectively to perturbations in the cellular environment. Recent findings have highlighted the key role of the ubiquitin-proteasome pathway for rapid clearance from cells of a number of proteins that control cell cycle, many of which are intrinsically unstable or at least partially disordered. In some cases, the degradation of unfolded or natively unfolded proteins such as tau, α-synuclein and p21WAF1/CIP1 may not require polyubiquitination but can be accomplished by the 20S proteasome in the absence of the regulatory 19S complexes [152, 153, 154, 155]. The intracellular levels of these proteins, including securin, cyclin B, cyclin E and p53, appear to be controlled by the ubiquitin-proteasome pathway, whose malfunctioning may lead to the loss of regulatory control of the cell cycle resulting in cell proliferation and cancer [156, 157, 158]. Similar effects can result from mutations that destabilise these proteins further; many mutations in the p53 gene result in cancer for this reason [157, 159].

Despite the structural adaptations discussed above, the propensity of natively unfolded proteins to aggregate can be significant; the aggregation of α-synuclein, for example, is associated with Parkinson's disease (see Table 1). Overexpression of this protein in transgenic mice has been shown to be followed by the appearance of ubiquitinylated inclusions and neural degeneration [160]; similarly, overexpression of tau in the central nervous system of transgenic mouse model is followed by the appearance of neurological symptoms [161]. Not surprisingly, increases in the levels of specific proteins, whether natively unfolded or not, in an organism can promote aggregation. Patients with Down's syndrome, for example, develop a form of Alzheimer's disease by the first decade or two of their life as a result of the overproduction of the Alzheimer precursor protein resulting from trisomy of chromosome 21 [118]. In general, an increase in the concentration of a protein favours its aggregation. It has recently been reported that the gain of toxicity by ataxin-1, a protein commonly associated with poly(Q) expansions results from an enhanced level of the wild-type protein in *Drosophila* models [162]. More recently it has been proposed that the appearance in the cytosol of minute amounts of misfolded PrP^{c} molecules following retrograde transport from ER and reduced degradation by proteasome is inevitably followed by cell death, even in the absence of the conversion to the self-propagating PrP^{sc} that appears not to be the primary toxic species [163]. In a related example, amyloid deposits of β_2-microglobulin in patients exposed to long-term dialysis seem to result principally from the high levels of this protein circulating as a result of dialysis. Indeed, for β_2-microglobulin, under equilibrium conditions a partially folded intermediate is significantly populated; the latter displays high propensity to aggregate and is stabilised by copper ions released from many types of dialysis membranes [164]. A recent crystal structure of monomeric human β_2-microglobulin has provided a possible explanation of its high amyloidogenicity by revealing clues about structural modifications favouring edge-to-edge interaction of the monomers [165].

The toxicity of pre-fibrillar aggregates discussed above further highlights the significance of the evolutionary development of the complex machinery responsible for both the unfolded protein response in the ER and the heat shock response in the cytosol; these cellular pathways undoubtedly prevent the intracellular accumulation of potentially toxic materials such as misfolded proteins or their early aggregates [152, 153, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174]. Indeed, a great deal of data reported in the past few years underscore the fundamental importance of the ubiquitin-proteasome pathway and the molecular chaperones (comprising Hsps and many other chaperone proteins such as crystallins, prefoldin, Hsc70 and ER chaperones such as BiP) in enabling cells to clear misfolded or damaged proteins, thus avoiding formation of toxic aggregates. The central role performed by molecular chaperones (notably Hsp90 and the Hsp70/40 system) in preventing the appearance of protein aggregates or in reducing ROS levels, has been shown both in cultured cells [166, 167, 168] and in model organisms such as yeast, transgenic mice and *Drosophila* [32, 170, 171, 172]. In addition, there is increasing evidence that indicates that malfunctioning of the Hsp and ubiquitin-proteasome systems is an important etiopathogenic factor in the development of a range of neurodegenerative conditions. These include Parkinson's and Alzheimer's diseases [29, 30, 172], the poly(Q) diseases [173, 174], several forms of retinitis pigmentosa [175], pseudoexfoliation syndrome [176], heredo-oto-ophthalmo-encephalopathy [177] and, possibly, systemic amyloidoses [178] as well as cataract,

either inherited and, possibly, associated with ageing [179, 180]. In all these cases the reported data depict a scenario in which Hsps, particularly the Hsp70/40 system, and the ubiquitin-proteasome degradation pathway, are actively involved in detoxifying misfolded proteins by stimulating their refolding or degradation.

If the molecular chaperone binding capacity were to be overwhelmed, this 'chaperone overload' would allow the accumulation in cells of unchaperoned misfolded proteins and permit their aggregation. This process would further impair proteasome activity leading to the appearance, at the centrosome, of inclusion bodies, known as aggresomes, which are assemblies of aggregated proteins, along with Hsps, ubiquitin and proteasome subunits [181]. The central role of molecular chaperones in the control of protein aggregation is further emphasised by recent data describing a number of 'chaperonepathies' [182]. These are rare inherited diseases whose pathogenicity arises from a reduced intracellular activity of chaperone proteins due to mutations in, or abnormal expression of, the genes encoding molecular chaperones [182]. Abnormal levels of molecular chaperones have recently been found in the brains of patients suffering from Alzheimer's disease [183] leading to the suggestion that abnormalities in the regulation of the genes for molecular chaperones should be investigated for all protein deposition diseases [182]. Finally, progress in understanding the exact role of the ubiquitin-proteasome system in intracellular proteolysis has advanced rapidly in the past few years and indicates that this pathway is indeed central to the regulation of cellular homeostasis. In effect, many of the major human degenerative diseases can collectively be described as 'ubiquitin protein catabolic disorders', where anomalous protein turnover resulting from defects in the ubiquitin-proteasome system can cause or contribute to the progression of these diseases ([184] and references therein).

Concluding remarks

The data reported in the past few years strongly suggest that the conversion of normally soluble proteins into amyloid fibrils and the toxicity of small aggregates appearing during the early stages of the formation of the latter are common or generic features of polypeptide chains. Moreover, the molecular basis of this toxicity also appears to display common features between the different systems that have so far been studied. The ability of many, perhaps all, natural polypeptides to 'misfold' and convert into toxic aggregates under suitable conditions suggests that one of the most important driving forces in the evolution of proteins must have been the negative selection against sequence changes that increase the tendency of a polypeptide chain to aggregate. Nevertheless, as protein folding is a stochastic process, and no such process can be completely infallible, misfolded proteins or protein folding intermediates in equilibrium with the natively folded molecules must continuously form within cells. Thus mechanisms to deal with such species must have co-evolved with proteins. Indeed, it is clear that misfolding, and the associated tendency to aggregate, is kept under control by molecular chaperones, which render the resulting species harmless assisting in their refolding, or triggering their degradation by the cellular clearance machinery [166, 167, 168, 169, 170, 171, 172, 173, 175, 177, 178].

Misfolded and aggregated species are likely to owe their toxicity to the exposure on their surfaces of regions of proteins that are buried in the interior of the structures of the correctly folded native states. The exposure of large patches of hydrophobic groups is likely to be particularly significant as such patches favour the interaction of the misfolded species with cell membranes [44, 83, 89, 90, 91, 93]. Interactions of this type are likely to lead to the impairment of the function and integrity of the membranes involved, giving rise to a loss of regulation of the intracellular ion balance and redox status and eventually to cell death. In addition, misfolded proteins undoubtedly interact inappropriately with other cellular components, potentially giving rise to the impairment of a range of other biological processes. Under some conditions the intracellular content of aggregated species may increase directly, due to an enhanced propensity of incompletely folded or misfolded species to aggregate within the cell itself. This could occur as the result of the expression of mutational variants of proteins with decreased stability or cooperativity or with an intrinsically higher propensity to aggregate. It could also occur as a result of the overproduction of some types of protein, for example, because of other genetic factors or other disease conditions, or because of perturbations to the cellular environment that generate conditions favouring aggregation, such as heat shock or oxidative stress. Finally, the accumulation of misfolded or aggregated proteins could arise from the chaperone and clearance mechanisms becoming overwhelmed as a result of specific mutant phenotypes or of the general effects of ageing [173, 174].

The topics discussed in this review not only provide a great deal of evidence for the 'new view' that proteins have an intrinsic capability of misfolding and forming structures such as amyloid fibrils but also suggest that the role of molecular chaperones is even more important than was thought in the past. The role of these ubiquitous proteins in enhancing the efficiency of protein folding is well established [185]. It could well be that they are at least as important in controlling the harmful effects of misfolded or aggregated proteins as in enhancing the yield of functional molecules. Interestingly, the ideas of molecular chaperones emerged from the study of the effects of heat shock, where expression of this type of proteins is increased in order to counter the effects of protein unfolding and aggregation. The role of the molecular chaperones in enhancing protein folding emerged from the realisation that they are essential, albeit often at much lower levels, in normally functioning cells where they are estimated to account for about 1% of the total

protein content [28]. This observation is equally important for their role in reducing the harmful effects of misfolded and aggregation-prone species. Hence a 'chaperone overload' is very likely to be associated with failure to control the accumulation of toxic forms of proteins [186].

Such considerations have also recently led to the hypothesis that molecular chaperones can act as capacitors of morphological evolution due to their ability to conceal by their binding, and hence to render harmless, mutations that could otherwise be damaging to the cell. Evidence for this idea has come from studies of *Drosophila* [187, 188], yeast [189] and *Arabidopsis* [190] and could well be important even in humans. The latter function of molecular chaperones could be of very considerable importance as it would reduce the constraints on protein evolution resulting from random mutations imposed by the intrinsic potential of many such mutations to enhance the tendency of the affected protein to aggregate. Moreover, the probability of both the phenotypic manifestation of such mutations and the nucleation of aggregation should increase during ageing [95]. Under such conditions modifications of the intracellular milieu are likely to occur, damaged proteins and their polyubiquitinylated derivatives tend to accumulate, and the induction of chaperones, together with the efficiency of the proteasome and the ubiquitinating/deubiquitinating enzymes, is likely to be impaired. Such conditions would undoubtedly increase the probability of overwhelming the ability of chaperones to act as effective 'housekeeping' agents and thus lead to a sudden build-up of protein aggregates within the cell. Such a situation would in turn cause further problems by impairing the function of the ubiquitin proteasome system in a type of positive feedback mechanism or autocatalytic cycle. The latter could explain the precipitous loss of neuronal function that frequently characterises the progression of many neurodegenerative diseases.

Extension of these ideas has led to another interesting hypothesis, that a larger number of degenerative and other diseases than is presently known results from the presence in cells of even minute amounts of aggregates that produce significant impairment of cell viability without a clear amyloid phenotype [50]. The presence of such aggregates could result, for example, from general effects of ageing such that the cellular clearance machinery becomes less efficient and the propensity to aggregate enhanced by processes such as chemical modification (e.g. as a result of oxidative stress). Indeed, the number of diseases associated with misfolding and aggregation is increasing steadily [175, 176, 191, 192, 193, 194]. In addition, amyloid deposits are frequently found in gastrointestinal vessels, although the pathogenesis and clinical significance of such findings are as yet unclear [195].

The increasing prevalence of amyloid diseases in general is undoubtedly associated with recent increases in human life expectancy, particularly in highly developed countries, as a result of the control of many infectious diseases and improved hygiene. One can consider that we are beginning to see the limitiations of the effects of proteins being optimised to resist aggregation simply as a result of evolutionary pressure [148]. The latter is limited by our lifespans, or more probably by the age at which we typically pass on our genes. It is also interesting, however, that several outbreaks of transmissible amyloid diseases, associated with prions have occurred in recent times, most dramatically that in the United Kingdom involving bovine spongiform encephalopathy, or 'mad cow disease'. This epidemic is almost universally accepted to have been the result of recent practices such as the feeding of young cows with the remains of old ones. Such a situation is analogous to the outbreak of kuru in Papua New Guinea in the 1950s which was associated with ritual cannibalism, and of Creutzfeldt-Jakob disease in individuals treated with contaminated growth hormone purified from human cadavers. The prion diseases are thought to be triggered by the ingestion of aggregated tissue, and the circumstances of such outbreaks reflect the violation of practices to which evolutionary pressure has applied [148]. In addition, at least one example of an amyloid disease is associated with a modern medical procedure, haemodialysis, which causes a specific protein, β_2-microglobulin, to aggregate. The amyloid conditions have indeed been called 'civilisation' or 'post-evolutionary' diseases as they have become prevalent as a result of our recent abilities to prolong our lifespans or of the introduction of new medical and agricultural practices [148, 196, 197]. This review focusses on the nature and origins of misfolding and aggregation diseases. Although it is beyond the scope of this contribution, we draw attention to the development of a wide range of new therapeutic strategies many of which are firmly based on the rapidly evolving knowledge of the diseases that we have outlined [11, 64, 197, 198, 199]. One can therefore be optimistic that our rapidly developing scientific and medical skills will be able to find ways through which we can avoid or treat many of these conditions in the future.

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