

Review

Protein misfolding and aggregation: new examples in medicine and biology of the dark side of the protein world

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Abstract

The data reported in the past 5 years have highlighted new aspects of protein misfolding and aggregation. Firstly, it appears that protein aggregation may be a generic property of polypeptide chains possibly linked to their common peptide backbone that does not depend on specific amino acid sequences. In addition, it has been shown that even the toxic effects of protein aggregates, mainly in their pre-fibrillar organization, result from common structural features rather than from specific sequences of side chains. These data lead to hypothesize that every polypeptide chain, in itself, possesses a previously unsuspected hidden dark side leading it to transform into a generic toxin to cells in the presence of suitable destabilizing conditions. This new view of protein biology underscores the key importance, in protein evolution, of the negative selection against molecules with significant tendency to aggregate as well as, in biological evolution, of the development of the complex molecular machineries aimed at hindering the appearance of misfolded proteins and their toxic early aggregates.

These data also suggest that, in addition to the well-known amyloidoses, a number of degenerative diseases whose molecular basis are presently unknown might be determined by the intra- or extracellular deposition of aggregates of presently unsuspected proteins. From these considerations one could also envisage the possibility that protein aggregation may be exploited by nature to perform specific physiological functions in differing biological contexts. The present review focuses the most recent reports supporting these ideas and discusses their clinical and biological significance.

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1. Introduction

Protein misfolding and aggregation is one of the most exciting new frontiers in protein chemistry as well as in molecular medicine. The current interest in this topic arises from several considerations; it is thought that the knowledge of the molecular basis of protein misfolding and aggregation may help to elucidate the physicochemical features of protein folding; it is also expected to shed light on the molecular and biochemical basis of a number of

pathological conditions of dramatic social impact such as Alzheimer's and Parkinson's diseases, type 2 diabetes, cystic fibrosis, some forms of emphysema and others. The common hallmark of such degenerative diseases is the presence, in the affected tissues and organs, of proteinaceous deposits that, in most cases, are believed to represent the main causative agents of the clinical symptoms [1–3]. A group of roughly 20 protein deposition diseases, usually referred to as amyloidoses, are characterized by the presence of deposits of fibrillar aggregates found as intracellular inclusions or extracellular plaques (amyloid) whose main constituent is a specific peptide or protein, different in the varying diseases (Table 1). Despite the structural and chemical differences of the polypeptide

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Table 1

A summary of the main amyloidoses and the proteins or peptides involved^a

Clinical syndrome	Fibril component
<i>Alzheimer's disease</i>	<i>Aβ peptides (1–40, 1–41, 1–42, 1–43); Tau</i>
<i>Spongiform encephalopathies</i>	<i>Prion protein (full-length or fragments)</i>
<i>Parkinson's disease</i>	<i>α-synuclein (wild type or mutant)</i>
<i>Fronto-temporal dementias</i>	<i>Tau (wild type or mutant)</i>
<i>Familial Danish dementia</i>	<i>ADan peptide</i>
<i>Familial British dementia</i>	<i>ABri peptide</i>
<i>Hereditary cerebral haemorrhage with amyloidoses</i>	<i>Cystatin C (minus a 10-residue fragment); Aβ peptides</i>
<i>Amyotrophic lateral sclerosis</i>	<i>Superoxide dismutase (wild type or mutant)</i>
<i>Dentatorubro-pallido-Luysian atrophy</i>	<i>Atrophin 1 (polyQ expansion)</i>
<i>Huntington disease</i>	<i>Huntingtin (polyQ expansion)</i>
<i>Cerebellar ataxias</i>	<i>Ataxins (polyQ expansion)</i>
<i>Kennedy disease</i>	<i>Androgen receptor (polyQ expansion)</i>
<i>Spino cerebellar ataxia 17</i>	<i>TATA box-binding protein (polyQ expansion)</i>
Primary systemic amyloidosis	Ig light chains (full-length or fragments)
Secondary systemic amyloidosis	Serum amyloid A (fragments)
Familial Mediterranean fever	Serum amyloid A (fragments)
Senile systemic amyloidosis	Transthyretin (wild-type or fragments thereof)
Familial amyloidotic polyneuropathy I	Transthyretin (over 45 variants or fragments thereof)
Hemodialysis-related amyloidosis	β 2-microglobulin
Familial amyloid polyneuropathy III	Apolipoprotein A-1 (fragments)
Finnish hereditary systemic amyloidosis	Gelsolin (fragments of the mutant protein)
Type II diabetes	Pro-islet amyloid polypeptide (fragments)
Medullary carcinoma of the thyroid	Procalcitonin (full-length or fragment)
Atrial amyloidosis	Atrial natriuretic factor
Lysozyme systemic amyloidosis	Lysozyme (full-length, mutant)
Insulin-related amyloid	Insulin (full-length)
Fibrinogen α -chain amyloidosis	Fibrinogen (α -chain variants and fragments)

^a Conditions affecting the central nervous system are written in italic.

chains aggregating into amyloid, amyloid fibrils are surprisingly similar in their appearance and structural features (increased content of beta structure) and tinctorial properties (binding of dyes such as thioflavin T and Congo red, see later).

In addition to amyloidoses, other protein misfolding diseases with deposition of protein aggregates which are not amyloid in nature have been known for a long time. Serine protease inhibitors such as α 1-antitrypsin, antithrombin and plasminogen activator inhibitor 1 may be destabilized by specific mutations. As a consequence, the exposed mobile reactive loop inserts, as an extra beta-strand, into the beta-sheet of another identical molecule; when propagated, these structural modifications lead to the formation of protein

polymers that are retained intracellularly, leading to cell impairment by a loss of function (the lack of active protein) and a toxic gain of function (the cytotoxicity of protein aggregates) (reviewed in Ref. [4]). In α 1-antitrypsin deficiency, 1 out of over 70 α 1-antitrypsin mutants aggregate into the endoplasmic reticulum of hepatocytes leading to liver disease. In addition, the lack of active protein leaves lung parenchyma unprotected against the enzyme neutrophil elastase leading to early-onset emphysema (reviewed in Ref. [4]).

The studies performed in the last few years have led to a reappraisal of the theme of protein misfolding and aggregation. Presently, it is thought that protein aggregation is a much more widespread phenomenon than previously believed involving a higher number of peptides and proteins than those found in the amyloid aggregates that characterize the known amyloid diseases (reviewed in Ref. [5]). A number of reports indicate that most (possibly any) proteins and peptides are able to aggregate into amyloid assemblies under suitable destabilizing conditions (see below); thus protein aggregation is presently considered a pathway alternative to protein folding where intermolecular, rather than intramolecular, interactions are favoured.

The idea that protein aggregation may be intrinsic to the common peptide backbone of any polypeptide chain suggests that protein evolution must have faced this previously unappreciated constraint; indeed, any functional sequence endowed with a tendency to aggregate under the medium conditions where it performs its biological function must have been discarded in order to provide cells with functional and stable proteins. In addition, the generic tendency of polypeptide chains to aggregate highlights the importance and the significance of the evolution of the complex molecular machineries ensuring the quality control of protein folding. The latter comprise molecular chaperones both in the cytosol (heat-shock proteins, crystallins, prefoldin, Hsc70,) and in the endoplasmic reticulum (Bip, Grp94, calnexin) and the ubiquitin–proteasome pathway. The main physiological function of these machineries is to favour folding of polypeptide chains and to avoid inappropriate interactions of polypeptides misfolded or unable to promptly fold into the correct three-dimensional structure and, when this task is not achieved, to promote their degradation.

The high efficiency of such a folding quality control allows a significant percentage of the proteins maturing in the ER to be cleared before they can properly fold [6,7]. This may be beneficial, improving the promptness of the immune response against viral infections [7] but may also have adverse effects. For example, the most frequent mutation of the CFTR chloride channel associated with cystic fibrosis (Δ F508CFTR) interferes with the correct folding of this polypeptide chain (which would still be active when folded), leading the ER quality control machinery to clear it (Ref. [8] and references therein).

The fundamental importance of the molecular chaperones is further testified by a number of recently described diseases due to mutations affecting the activity of specific chaperones (chaperonopathies) [9] or the efficiency of the ubiquitin–proteasome pathway (ubiquitin protein catabolic disorders) [10]. Indeed, some of these diseases display the features of specific amyloid diseases further stressing the close link between protein misfolding, aggregation and clinical symptoms of amyloid diseases [11–14].

In addition to these intracellular quality controls, others are found at the cell membrane or in the extracellular spaces and fluids. These comprise proteases such as neprilysin and IDE, which have been shown to digest A β and other aggregate precursors in their monomeric form but also as aggregates [15–17], and chaperones present at significant levels in extracellular fluids such as clusterin [18]. Evidence has been published that clusterin affects amyloid formation in vitro [19]. Although the mechanisms by which these extracellular proteases and chaperones could influence protein misfolding disease are yet to be established, they appear to be of importance in the management of extracellular protein deposits by higher organisms.

Presently, in the protein deposition diseases, the presence of aggregated material is believed to be the

cause, not a consequence of the clinical symptoms and the latter, at least in the neurodegenerative diseases, can ultimately be traced back to the toxic effects of the aggregates to the cells (what is known as the amyloid hypothesis) [1–3]. In the case of the peripheral amyloidoses, the presence of the aggregates, often found in very huge amounts, may by itself damage organs simply by hindering a proper flow of nutrients to the cells thus impairing tissue functions [20].

Many authors believe that the shared structural features of the amyloid aggregates both at the level of protofibrils and of mature fibrils are reflected into common early biochemical modifications in cells experiencing the presence of the toxic aggregates; these modifications eventually lead to the overwhelming and impairment of the defence mechanisms (notably chaperone proteins and the ubiquitin–proteasome pathway) resulting in cell death by apoptosis or necrosis. Indeed, a number of reports on early alterations of free calcium and reactive oxygen species in cells exposed to toxic aggregates or producing aggregating molecules seem to agree with this idea [21–28] (Fig. 1). The latter is also supported by a number of findings showing that annular, “doughnut”-shaped assemblies with a central pore are present among the heterogeneous population of pre-fibrillar aggregates of several different

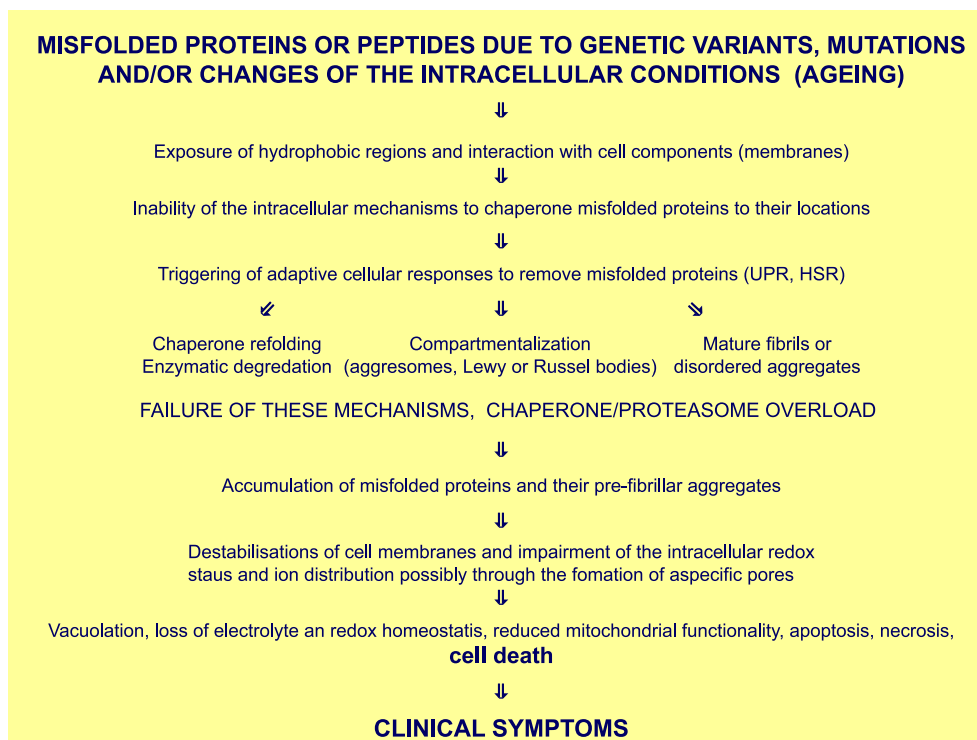


Fig. 1. Flow-chart of the molecular events leading misfolded polypeptides to induce cell death. The panel considers protein aggregation into mature fibrils as potentially beneficial since, at least in most cases, the true cytotoxic aggregates are the pre-fibrillar assemblies. The unfolded protein response (UPR) in the endoplasmic reticulum and the heat-shock response (HSR) in the cytosol are aimed at clearing misfolded proteins and their early aggregates. Cell death occurs as a consequence of a rise of misfolded/unfolded polypeptides and their toxic early aggregates overwhelming the chaperone–ubiquitin–proteasome clearing efficiency. The unstable, toxic pre-fibrillar aggregates may interact with cell membranes and impair their functions, resulting in modifications of ion distribution across them, possibly following aggregate organization into non-specific membrane pores. In most cases, increases in intracellular free Ca²⁺ and modifications of the redox potential (oxidative stress) are among the earliest biochemical alterations in exposed cells (modified from Ref. [60]).

protein and peptides [21,22,29–33]. These annular species are reminiscent of the pores formed by several bacterial pore-forming toxins as well as by some eukaryotic proteins, leading some authors to propose the “channel hypothesis” of amyloid aggregate toxicity [34,35].

The idea that protein aggregation may be a much more widespread process than previously believed either in medicine and in biology has recently gained support. Indeed, amyloid aggregates of specific mutant proteins have been found in an increasing number of familial degenerative pathologies of unknown origin (see Section 5). In addition, recent reports describe physiological functions of amyloid aggregates of specific proteins or peptides in particular biological systems as different as plants, bacteria and mammals, thus shedding a new light on the biological importance of protein aggregation (see Section 4).

2. Protein misfolding, aggregation and aggregate toxicity

In the case of protein deposition diseases of amyloid type, the molecular basis of protein aggregation is protein misfolding, where a specific polypeptide chain loses, or is unable to attain its native, closely packed three-dimensional structure, thus populating unfolded, partially folded or non-correctly folded states in equilibrium to each other. In these non-native states, the protein becomes loosely packed and its hydrophobic core becomes exposed to the solvent thus enhancing the tendency to nucleate initial oligomeric assemblies where the content of secondary beta structure is generally increased [1,2,36,37]. These “seeds” or “aggregation nuclei” provide a sort of template where other misfolded or partially folded molecules (or natively folded molecules in the case of the infectious prion diseases, see below) are recruited thus increasing the sizes of the growing assemblies eventually giving rise to fibrillar aggregates (reviewed in Ref. [5]).

2.1. Protein aggregation may result from several favouring conditions

The onset of aggregation may be triggered by any factor resulting in a rise of the concentration of the amyloidogenic precursor(s) such as a shift of the equilibrium between correctly folded and partially folded molecules towards the latter or an increase of the expression level of the affected protein and hence its whole equilibrium population comprising partially folded molecules (Fig. 2). This may be the case of mutations, environmental changes or chemical modifications reducing the conformational stability of the protein. Alternatively, specific mutations may enhance aggregation simply by favouring kinetically the assembly of the unfolded or partly folded monomers into the early oligomeric pre-fibrillar species (Fig. 2). In this aspect, recent data have shown that general physicochemical

features, such as mean hydrophobicity, net charge and propensity to alpha and beta structure formation, affect the tendency of an unfolded or partially folded polypeptide chain to aggregate [38]. This may explain the higher propensity to aggregation of peptides and natively unfolded proteins such as α -synuclein and tau carrying specific mutations enhancing their mean hydrophobicity or reducing their mean net charge. Intracellular aggregates of these proteins either wild-type and mutated, are the pathologic hallmark of the familial forms of synucleinopathies (Parkinson’s disease and others) and tauopathies (Alzheimer’s disease and others), respectively. A natively folded protein may also misfold and aggregate, provided it meets a suitable template favouring a specific conformational modification, as it is best exemplified by the prion diseases (Creutzfeldt–Jakob disease and others) where aggregates of the prion protein (PrP^{Sc}) recruit the natively folded PrP^C molecules, thus propagating the aggregating (PrP^{Sc}) structure [39]. This behaviour accounts for the transmissibility of the phenotypes determined by passing among individuals, even minute amounts of the PrP^{Sc} aggregates. Recent data suggest that other proteins/peptides are able to propagate a toxic conformation to the natively folded counterparts [40,41] (see also Section 5.7).

Finally, protein aggregation may be favoured under conditions resulting in the impairment or overwhelming of the molecular machineries aimed at performing the quality control of protein folding. The latter comprises the molecular chaperones either of the ER and the cytosol, the ER membrane carriers performing the retrograde transport of the proteins unable to fold in the ER lumen [42], the ATP-dependent proteolytic complexes in mitochondria and the components of the ubiquitin–proteasome pathway [43]. The data reported in the last few years highlight the central role performed by these machineries in ensuring that folding or unfolding intermediates are promptly bound and refolded by the chaperones or degraded by the ubiquitin–proteasome machinery so as their intracellular steady-state concentration is maintained at negligible levels [44]. Specific inactivating mutations of any of the components of the quality control or harsh environmental conditions such as heat shock, oxidative stress or chemical modification may impair the activity of the clearing machinery components and/or increase the number of misfolded or unfolded proteins the cells must face, resulting in the overwhelming of both the molecular chaperones and the proteasome (reviewed in Ref. [5]).

2.2. Amyloid fibrils share common structural features

Under conditions where it is destabilized, a protein or a peptide undergoes the path eventually leading to the appearance of mature amyloid fibrils. Despite the large differences in the structures of the proteins and peptides contributing to the aggregates found in the differing amyloidoses, amyloid fibrils are surprisingly similar and

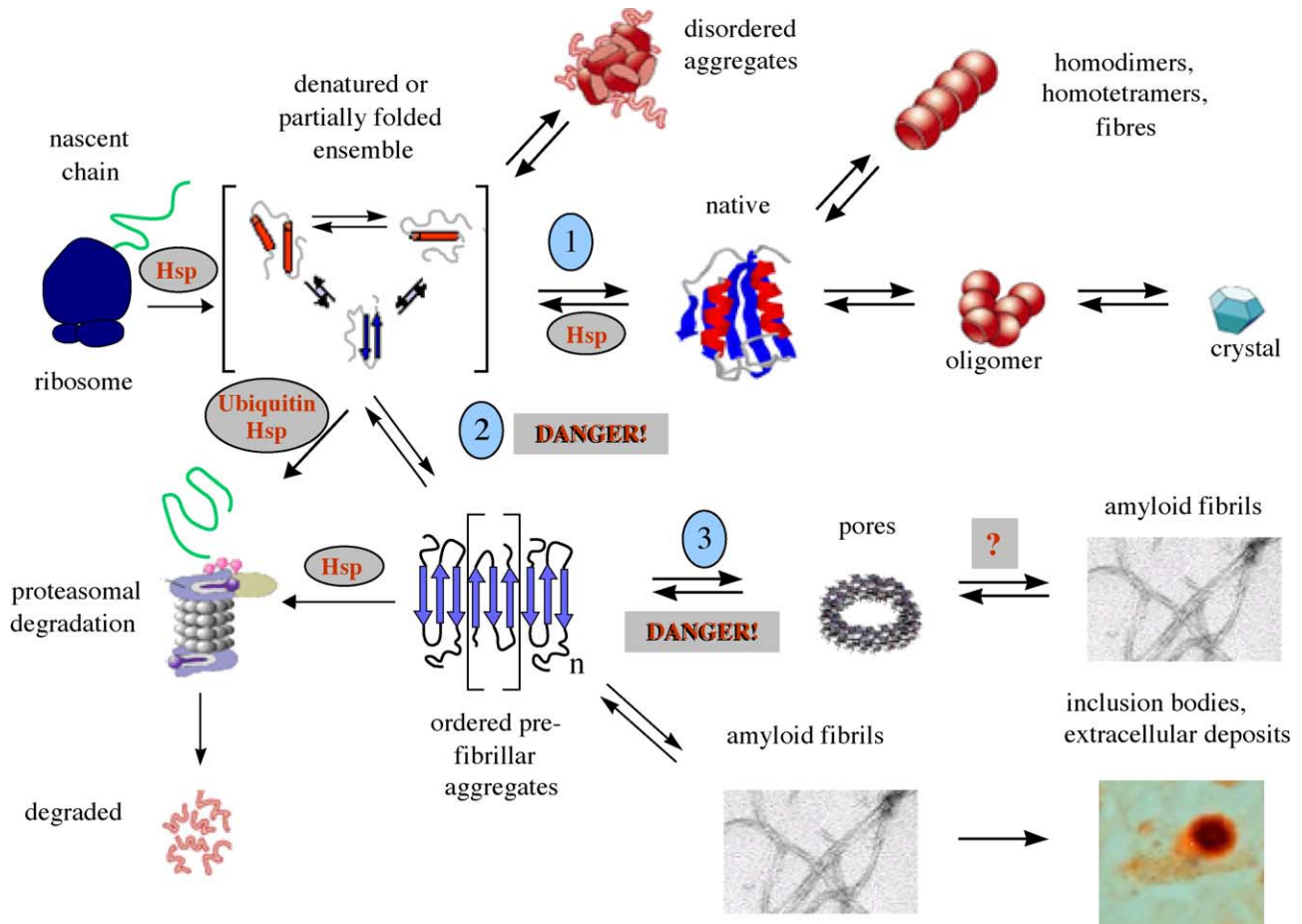


Fig. 2. The possible fates of newly synthesized polypeptide chains. Modifications of protein structure or medium conditions may favour protein–protein interactions into fibers or into crystalline lattices. Should these conditions be destabilising, the equilibrium ❶ is shifted to the left thus increasing the population of partly folded molecules. Under normal conditions, these are refolded by the molecular chaperones or cleared by the ubiquitin–proteasome machinery. Should these machineries be impaired or the population of misfolded molecules overwhelm their buffering possibility, disordered aggregates arise or the aggregation path is undertaken. Equilibrium ❷ is intrinsically shifted to the right and the nucleation of ordered aggregates is kinetically favoured by mutations increasing the mean hydrophobicity or propensity to beta structure or reducing the net charge of the misfolded/unfolded molecules. The formation of pre-fibrillar assemblies in the form of amyloid pores (equilibrium ❸) could be directly related to the cytotoxic effects of amyloids. The question mark indicates that it is not known whether amyloid pores (when formed) are on path or dead end intermediates of fibril formation. DANGER! indicates the processes generating the pre-fibrillar assemblies presently considered mostly associated with cell impairment. Molecular chaperones (heat-shock proteins and others) may suppress the appearance of pre-fibrillar aggregates by reducing the population of misfolded protein molecules assisting their correct folding or favouring their complete misfolding for proteasome degradation; they may also, to some extent, clear amyloid assemblies by detaching monomers and favouring their clearance. The data reported in the last few years support the idea that mature amyloid fibrils are substantially non-cytotoxic reservoirs of the pre-fibrillar, toxic assemblies (modified from Ref. [5]).

share basic structural features. Typically, amyloid fibrils are straight, unbranched, 6–12 nm wide (but larger in some cases) formed by a variable number of elementary filaments (protofilaments) around 1.5–2.0 nm in diameter, twisted around each other in a rope-like structure [45,46] (Fig. 3). These structural features have been studied by differing biophysical techniques such as transmission and cryo-electron microscopy, atomic force microscopy and solid-state NMR. Unfortunately, these techniques are unable to provide structural information at the atomic level; on the other hand, the fibrous and scarcely repetitive nature of the fibrils makes them unsuitable for investigation by X-ray diffraction. However, the latter technique has led to the

description of the ordered core of the amyloid fibrils as a cross-beta structure, where each protofilament results from a double row of beta-sheets provided by each monomer, whose strands run parallel to each other and perpendicular to the main fibril axis (Fig. 3). The cross-beta structure of the core of the amyloid aggregates is the main structural hallmark of the latter and is thought to be responsible for the tinctorial properties of these assemblies.

Recently, much interest has been focused on either the structural features of the pre-fibrillar intermediates preceding the appearance of the protofilaments and mature fibrils and the relationship between aggregate structure and toxicity. The studies reported in the last 5 years support

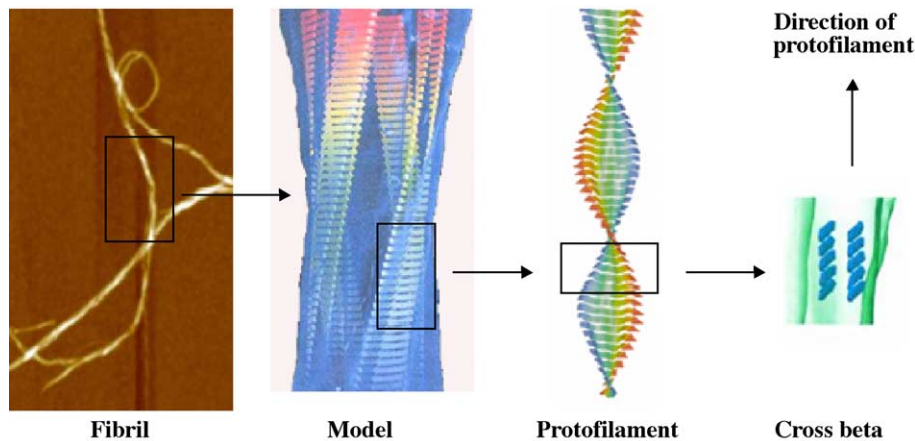


Fig. 3. Close-up view of the structural organization of an amyloid fibril. The four protofilaments are wound around each other and their core structure is a row of β -sheets where each strand runs perpendicular to the fibril axis (from Refs. [28,163]).

the notion that the pathogenic protein aggregates are the destabilised monomeric, or the non-fibrillar oligomeric, species of distinct morphology (protofibrils) preceding mature fibrils in the aggregation pathway. Protofibril appearance in tissues precedes the expression of the clinical phenotype thus explaining the lack of relationship found in most cases between extent of amyloid deposits and severity of the clinical symptoms [47,48]. The earliest protofibrils typically appear as globular assemblies 2.5–5.0 nm in diameter spontaneously organizing into chains and variously sized rings comprising small “doughnuts” with a central pore [28,49–56], further organising into ribbons, protofilaments and mature fibrils.

2.3. Amyloid pores may play a key role in aggregate toxicity

Despite the large number of reports that have appeared in the last few years on the molecular basis of cell impairment following exposure to amyloid aggregates, much must still be learned on the molecular, biochemical and biological features of the effects of the amyloid aggregates on living systems. Recently, a central role of the protofibrils has been proposed [49–52]. In most cases, these pre-fibrillar assemblies appear endowed with the highest toxicity, and a large body of evidence indicates that these are the true toxic species, whereas mature fibrils are much less toxic and can be considered as harmless reservoirs of the toxic assemblies [29,52,57–59] (see below).

Much interest has recently been raised by the possibility that a subpopulation of protofibrils, notably the amyloid pores, may account for the toxicity of the amyloid aggregates, as it has been shown for the aggregates of several different peptides and proteins, thus envisaging a basically common early biochemical mechanism of aggregate toxicity [60]. Since 1993, it was proposed the “channel hypothesis” of amyloid toxicity, whereby the

toxic aggregated species form non-specific pore-like channels in the membranes of the exposed cells [61] (Fig. 4). This behaviour is reminiscent of the action of several bacterial pore-forming toxins such as perfringolysin [62], but eukaryotic counterparts of this mechanism are also described. In mammals, for example, perforin, the C5b-8/9 complement assembly in the membrane attack complex and the BCL-2 family of pro-apoptotic proteins act by forming aspecific channels in the membranes of the target cells [63–65], although the amyloid nature of these channel has not been assessed. These similarities suggest the evolution of a death mechanism whereby protein oligomers act as biological “bullets” killing the target cells by forming non-specific membrane pores resulting in unbalance of the ion content.

Other hypotheses have been put forward to describe the biochemical basis of the toxicity of amyloid aggregates. Some refer to a number of data indicating that cells experiencing toxic aggregates undergo early changes of the intracellular ion content and redox status (reviewed in Ref. [5]). These data may be a consequence of the presence, in the exposed cells, of pores modifying membrane permeability; however, they could also follow some other type of membrane destabilization by the aggregates or the involvement of metal ions such as copper known to favour protein aggregation and oxidative stress. In addition, a number of alternative explanations have been reported for the toxicity of aggregates of proteins containing Gln expansions [66].

3. Could protein aggregation reflect a tendency inherent to all polypeptide chains?

The field of protein misfolding and aggregation has widened since 1998, when it was first shown that two proteins unrelated to any amyloid disease were able to

aggregate in vitro provided they were partially unfolded [67,68]. These findings demonstrated for the first time that protein aggregation was not a peculiar property of the amino acid sequences of the few polypeptide chains responsible for the formation of the aggregates found in the amyloid diseases; rather, even proteins found normally folded under physiological conditions can unfold and aggregate in vitro into assemblies undistinguishable from those formed in vivo by the proteins associated with the known amyloid diseases. Since then, an increasing number of proteins and natural or synthetic peptides not associated with disease (reviewed in Ref. [5]) and of amino acid homopolymers [69] have deliberately been made to assemble in vitro into fibrillar and pre-fibrillar aggregates undistinguishable from those found in vivo. This happens under partially destabilizing conditions (acidic pH values, high temperature, lack of ligands or moderate concentrations of salts or of co-solvents such as trifluoroethanol) where the tertiary interactions are destabilized, whereas the secondary contacts, notably hydrogen bonds, are still favoured. Under these conditions, the protein misfolds in a molten globule-like structure where the secondary interactions are substantially maintained but normally buried hydrophobic residues become solvent-exposed. The reduced physicochemical stability of the partially unfolded monomers leads them to organize into the oligomeric assemblies seen in the path of fibrillization and eventually into stable mature fibrils.

3.1. The potential for aggregation is inherent to the peptide backbone

These results provide strong support to the idea that protein aggregation is a rather common behaviour of the polypeptide chains possibly linked to the structure of their common peptide backbone, thus explaining the shared structural properties of the amyloid fibril core. On this regard, amyloid fibrils may be seen as the product of an ancestral generic tendency of the polypeptide chains to undergo beta structure-based intermolecular interactions arising from their peptide backbone [69]. Protein evolution must therefore have selected specific amino acid sequences suitable to attain folds able not only to perform efficiently specific biological functions but also to segregate at their interior the main chain atoms, avoiding the inherent tendency to interact with other polypeptide chains and to aggregate.

The evolved amino acid sequences of natural proteins must possess structural features favouring their biological activity but also their folding over aggregation under the conditions where each protein must perform its function (Fig. 5). Hence, the evolution of the highly cooperative nature of the functional protein structures appears to be a critical step in the appearance of proteins stable against their inherent tendency to aggregate for lengths of time consistent with their intracellular half-lives and with the overall reproductive life span of an individual [70]. The exposure

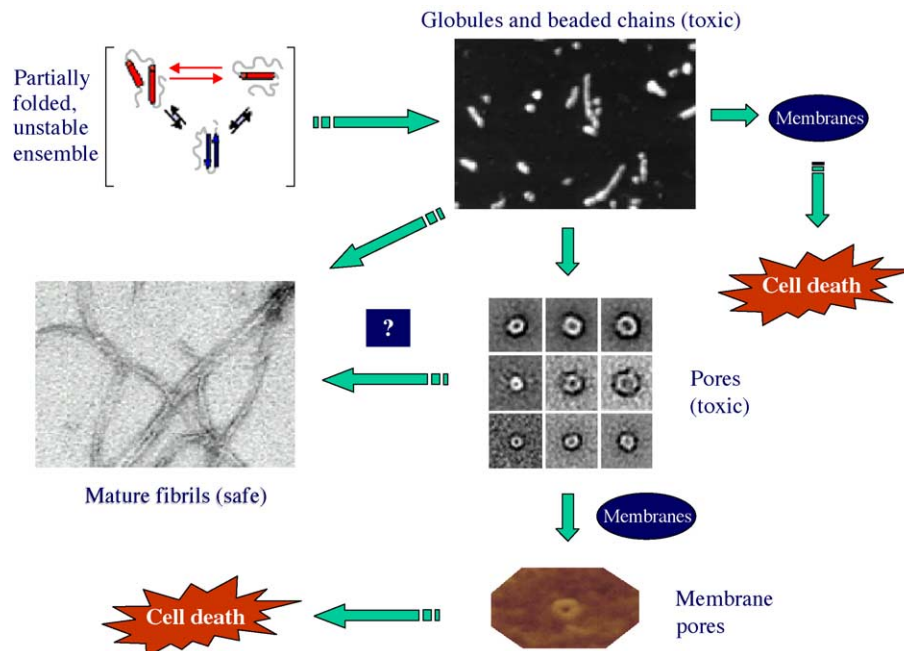


Fig. 4. The heterogeneous population of pre-fibrillar aggregates comprises globular assemblies further organising into beaded chains and doughnut-shaped entities currently associated with cytotoxicity due to their ability to interact with cell membranes. In particular, the pore-like assemblies may impair membrane permeability altering metal ion distribution between intracellular and extracellular media as well as among intracellular compartments triggering cell apoptosis. The question mark indicates that it is not known whether amyloid pores (when formed) are on path or dead end intermediates of fibril formation (modified from Ref. [5]). The electron micrographs are from Lashuel et al. [164] and from Harper et al. [165]. The AFM image is from Ding et al. [166].

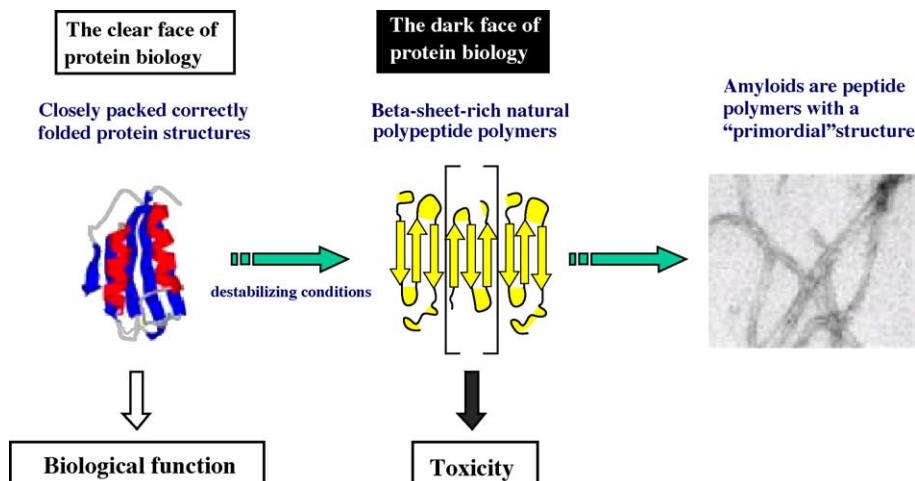


Fig. 5. The polypeptide chains display the intrinsic tendency to interact to each other with basic primordial features dominated by hydrogen bonds between main chain atoms, giving rise to polymers rich in β structure. Evolution has selected amino acid sequences able to organize into monomeric structures where the main chain is folded in a unique way and the closely packed side chains prevent its inherent tendency to generate primordial polymers. As the main chain is preserved as a common feature of all natural proteins, these can revert to their "primordial" tendency under conditions loosening the closely packed tertiary structure. Hence, natural proteins can be thought of as a special group of evolved polymers where the specific interactions among side-chains dictate the globular structure, whereas amyloid fibrils can be seen as the products of the intrinsic tendency of the polypeptide chains to organize into the "primordial" polymer structure. Hence, peptides and proteins possess a hidden dark face associated with their toxicity, resulting from the intrinsic tendency of the peptide backbone to polymerize into hydrogen bonded beta-sheet rich assemblies displaying the characteristic cross-beta structure of amyloid fibrils. This inherent tendency becomes evident when proteins are destabilized so as their closely packed tertiary structures are loosened and partially folded unstable molecules are significantly populated.

of the main chain atoms of polypeptide chains under partially destabilizing conditions would favour such a primordial behaviour inherent to the peptide backbone leading to the intermolecular interactions found in the amyloid aggregates.

Recently, possible models of the primordial structure determined by such an intrinsic behaviour of the peptide backbone have been proposed. These include the μ -helix [32] and the parallel β -helix resulting in some way from a collapse of the β -cylinder proposed by Perutz et al. [71,72] as a model for the structure of polyglutamine aggregates. The β -helix model seems to account for the experimental observations on the basic architecture of a variety of amyloid fibrils [73]. Recent database surveys have highlighted some of the evolutionary adaptations aimed at avoiding intermolecular association of β -strands in native proteins [74–76].

3.2. Protein aggregation may be a rather common event in cells

The intrinsic aggregability of polypeptide chains suggests that protein aggregation *in vivo* might be a rather common event and that one of the main functions of the molecular chaperones and the ubiquitin–proteasome pathway could be to refold or, alternatively, to clear misfolded proteins when they appear in the crowded intracellular milieu [44]. It also suggests that protein aggregation diseases following any unbalance between the rate of aggregate appearance and the efficiency of the clearing machineries might be more common than previously suspected. Therefore, it is con-

ceivable to expect that in the near future other degenerative conditions besides the known amyloidoses will be shown to be associated with deposition of aggregates of proteins presently not associated with disease (see Section 5). It is also possible to hypothesize that, in particular biological systems, specific protein aggregates may be produced with some physiological significance performing specific functions (see Section 4).

Recent reports show that disease-unrelated proteins such as the SH3 domain of the PI3 kinase, the N-terminal domain of the bacterial hydrogenase maturation factor HypF, human endostatin and apomyoglobin besides aggregating into assemblies identical to those produced by disease-associated peptides and proteins also display very similar cytotoxic effects. Even in this case, the protofibrils appear to be the assemblies endowed with the highest toxicity, whereas protofilaments and mature fibrils appear substantially non-toxic [77–80]. These data clearly indicate that, similarly to protein aggregation, even aggregate toxicity resides in the shared structural properties of protein aggregates. This conclusion is supported by a recent paper showing that antibodies raised against pre-fibrillar aggregates of A β peptides cross-react with similar aggregates, but not with mature fibrils, of other differing peptides and proteins such as amylin, α -synuclein, and the amyloidogenic prion fragment [81]. These findings highlight the presence, in these aggregates, of common structural features differing from those found in the mature fibrils. In addition, these antibodies were able to relieve the cytotoxicity of all the investigated pre-fibrillar aggregates in a non-specific way, thus supporting the idea that, as aggregation, even aggregate

toxicity depends on generic structural features rather than on specific amino acid sequences [81].

3.3. The dark side of the protein world

Overall, the aggregability and aggregate toxicity of most (possibly all) proteins sheds a new light on the protein world. Indeed, protein molecules besides being the fundamental molecular tools of the living world, also possess a “dark side” leading them to transform into rogues able to kill cells (Fig. 5).

The generic ability of peptides and proteins unrelated to disease to form amyloid aggregates with specific cytotoxic effects in their pre-fibrillar organization increases largely the number of molecular structures one can investigate to describe the general structural and physicochemical features underlying the molecular mechanisms of protein folding, misfolding and aggregation as well as the biochemical modifications in cells exposed to these aggregates. It also has important consequences for an in-depth understanding of the fundamentals of the origin of protein deposition diseases as well as very important practical outcomes. For example, protein engineers must take into account this behaviour when designing new or modified peptides and proteins. Moreover, the pharmacological strategies aimed at treating the various amyloidoses must re-consider their targets in order to develop molecules aimed at reducing the toxicity of pre-fibrillar aggregates or their formation, and hence the build-up of misfolded proteins, rather than at simply hindering the growth of mature fibrils (less toxic or even harmless).

4. Could protein aggregates or their precursors perform specific biological functions?

As suggested above, if protein aggregation is a generic property of polypeptide chains under suitable conditions, one can expect that somewhere in the living world this behaviour has been exploited by specific systems to perform specialized functions. This raises the question as to whether amyloid aggregates may have physiological significance in some instances. Until a couple of years ago, it was commonly believed that protein aggregation was a drawback of protein behaviour, a rare accident in the normal path of protein folding with clinical consequences and devoid of any physiological significance. However, recent findings highlight the possibility that, in certain instances, amyloid aggregates may perform specific biological functions (Table 2). Indeed, this is not surprising if we consider protein aggregation a possible behaviour of any polypeptide chain. Nature exploits every possibility given by natural processes to perform meaningful activities. In this view, amyloid formation could also be considered a biological pathway that has been conserved

Table 2

Physiological functions of amyloid protofilaments or fibrils in specific biological systems

Protein	Source	Function	Reference
Bacterial toxins?	bacteria	cell killing	[62]
Curlin	<i>E. coli</i>	substrate adhesion	[83]
Chaplins	<i>S. coelicolor</i>	aerial hyphae formation	[86]
HET-s prion	<i>P. anserina</i>	self/non-self recognition	[88]
Hydrophobins?	fungi	fungal coat	[87]
Chorion proteins	silkworm	egg and embryo protection	[89,90]
Crystallins	mammals	refractive index of the lens	[93]
Pore proteins?	mammals	cell killing/apoptosis	[58–60]
SAA?	mammals	cell killing	[91,92]
A β peptides	human	small cerebral vessel sealing	[94]
Pmel17 (fragment)	human	melanine polymerization	[96]

in evolution to produce natural product nanostructures [82].

4.1. The microbial world offers examples of amyloid aggregates with biological functions

A paper appeared in 2002 first showed that the β -sheet-rich, highly aggregated fibers known as curli produced by *Escherichia coli* were indeed amyloid fibrils morphologically identical to those described in amyloid diseases [83]. Curli fibrils are deposited at the cell surface, where they favour cell adhesion to the substrate in the colonization of inert surfaces and mediate the binding to various host proteins [83,84]. These fibrils arise from a protein, curlin, through a complex nucleation–precipitation process requiring chaperone-like and nucleator proteins encoded by the *csgAB* and *csgDEFG* operons whose function is possibly to prevent curlin polymerization within the cell while accelerating it at the cell surface [85].

This finding raises the possibility that in other cases the proteinaceous filaments found at the cell surfaces of other bacteria may be amyloid. Indeed, more recently, it has been reported that the formation of aerial hyphae in *Streptomyces coelicolor* requires the assembly into amyloid-like fibrils of a novel class of secreted hydrophobic highly surface-active proteins (chaplins), although more convincing proof of their amyloid nature must be provided [86]. These proteins lower the water surface tension of the aqueous environment by forming amyloid-like fibrils, thus enabling hyphae to grow into the air and also provide the aerial structures with a hydrophobic coat. It appears possible that even in other fungal systems, hyphae may be coated with amyloid-like fibrils of proteins performing the same function as chaplins. For example, chaplins resemble the hydrophobins of filamentous fungi, a group of proteins assembled in highly insoluble films composed of a mosaic of amyloid-like fibrils that help hyphae to

breach the water–air interface by the same mechanism [87]. It has also been reported that the HET-s prion protein of the filamentous fungus *Podospora anserina*, aggregating into amyloid fibrils, performs some function associated with heterokaryon incompatibility and hence in the self/non-self recognition [88].

As mentioned above, some bacterial pore-forming toxins kill the target cells by assembling into pore-like oligomers that penetrate the plasma membrane permeabilizing the cell that eventually dies. In some cases, protein insertion into the plasma membrane of the target cell requires a conformational rearrangement leading to changes in the secondary structure elements. For instance, perfringolysin inserts into the plasma membrane of the target cell upon conversion of six alpha-helices in domain 3 into four amphipathic beta-strands [62]. It is not known whether such a change may be equivalent to those leading other proteins to assemble into true pre-fibrillar amyloid structures, however, it envisages an overall mechanism similar to that proposed for the toxic effects of the pre-fibrillar aggregates of amyloid type.

4.2. Protein aggregates with specific functions are found even in higher organisms

The possible physiological exploitations of specific amyloid aggregates are not restricted to the microbial world; even in higher organisms such as insects and mammals examples of possible physiological functions of amyloids have been reported. Recently, a peptide from the A- and B-family of the silkworm chorion proteins has been found to account for 30% of the proteinaceous material found as amyloid aggregates in the eggshell, leading to the suggestion that silkworm chorion is a natural amyloid with protective properties necessary for survival and development of the oocyte and embryo [89,90]. Recently, it has been shown that acute-phase isoforms of human and murine serum amyloid A (SAAp and SAA2.2, respectively) are present in solution as hexameric assemblies with a central pore and that these channels are able to permeabilize synthetic and cell membranes, although it is not clear whether these hexamers show the cross-beta structure of amyloids [91]. SAA concentration in plasma rises dramatically during the onset of an acute inflammation and this increase is maintained during chronic inflammation [92]. Such an increase is believed to contribute to the occasional development of reactive amyloidosis (amyloid A amyloidosis) where the whole protein and its 1–76 N-terminal fragment (AA) are found to form amyloid deposits in the kidney, liver and spleen [92]. It has been proposed that the toxic effects of the SAA and AA aggregates are a drawback of the proposed physiological function of the hexameric assemblies: to form toxic channels on the cell membranes of the invading bacteria thereby protecting against infection [91].

Many reports that have appeared in the past few years have shown that the major proteins in normal mammalian lenses exist predominantly as β -pleated sheets *in vivo*; more recently, it has been reported that in normal lenses, a positivity does exist to the classical amyloidophilic stains such as Congo red and thioflavine T, supporting the idea that in the interior fiber cells of the mammalian lens, protein β -sheet arrays are physiologically organized in an amyloid-like supramolecular order [93]. In this case, evolution could have exploited the inherent high stability of amyloids to ensure the long-term structural integrity and transparency of the lens.

A large body of literature supports the idea that A β aggregates are key elements in the process of sealing capillaries and arterioles or in maintaining regional integrity following traumatic insults (reviewed in Ref. [94]). APP is an acute phase reactant up-regulated in response to inflammation as well as to a number of cellular stresses such as energy shortage, oxidative stress and calcium dysregulation; the latter also appear to favour the production of amyloidogenic APP derivatives by modifying APP processing [95]. Indeed, the rapid deposition of A β following severe head trauma highlights the possible involvement of these aggregates in maintaining vascular integrity thus protecting brain parenchyma against haemorrhage. These data question the validity to remove A β from brain as in the immunologic approach against Alzheimer's disease [94].

More recently, it has been reported that melanine granule polymerization in melanosomes requires the presence, in the latter, of intraluminal fibrous striations. These are formed by the polymerization of a luminal domain fragment specifically cleaved from the resident membrane protein Pmel17. It has been suggested that, besides providing the substrate onto which melanine polymerization occurs, Pmel-17 cleavage regulates melanosome biogenesis by exploiting its fibrillogenic potential [96]. This has led to propose that this mechanism could be shared with other tissue-specific organelle structures, helping to clarify the molecular mechanisms governing their biogenesis as well as the formation of their unique structural features [96].

In addition to these examples, it must be reminded that, as mentioned above, a number of mammalian proteins comprising perforin and the membrane attack complex made by the C5b-8/9 of the activated complement generate physiologically oligomeric assemblies endowed with toxic properties [62–64]. The formation of the latter requires partial unfolding of the involved proteins and exposure of hydrophobic surfaces leading to the formation of ring-shaped complexes that insert deeply into the lipid bilayer of the membranes of the target cells where they form small “leaky” pores. Even in this case, it is not known whether the resulting inter-subunit association requires structural modifications similar to those found in proteins assembling into amyloid aggregates.

4.3. Folding variants of specific proteins display toxicity specifically to tumoural cells

Recent reports highlight a specific cytotoxic effect on tumoural cells of a partially unfolded state of the milk protein α -lactalbumin, which forms at acidic pH values, where the protein loses its bound calcium ions adopting the apo state; the latter possesses a high-affinity fatty acid binding site containing a bound oleic acid molecule that stabilizes and traps the altered protein conformation (reviewed in Ref. [96]). This folding variant of α -lactalbumin, possibly displaying molten-globule-like features, displays bactericidal activity [97] and induces an apoptotic-like death in a large number of differing types of tumour cells, whereas leaving fully differentiated cells unaffected. The findings on α -lactalbumin suggest that protein folding variants, whose formation in peripheral tissues may depend on changes of the conditions affecting the folding features, may perform specific biological functions; they have also led to the proposal of a protective effect of breastfeeding against childhood tumours and the use of this folding variant of α -lactalbumin as a possible new anti-cancer tool [97].

Indeed, 15 years ago it was first proposed that the molten globule states of proteins are biologically important in allowing proteins to cross cell membranes [98], suggesting that protein conformational states that are currently believed to be precursors of amyloid aggregates may, at least in some instances, exploit biological functions, raising the question as to whether this behaviour could be more widespread in biological systems. Similar results have recently been reported with apoptin, an apparently disordered protein encoded by chicken anaemia virus forming remarkably stable globular aggregates only in tumoural cells, whereas the intracellular environment of the healthy cells seems to prevent the cytoplasmic accumulation of such aggregates [99]. However, it has not been assessed whether the apoptin toxic aggregates are of amyloid type.

5. Protein deposition diseases might be much more frequent than previously suspected

The increasing prevalence of protein deposition diseases in humans is primarily associated with the increasingly higher life expectancy, particularly in developed countries. Proteins are not necessarily optimized to maintain their correctly folded states under progressively declining environmental conditions and impaired biological safety mechanisms as it happens during ageing. Therefore, it is likely that we are starting to experience the work of protein evolution in optimizing the resistance of proteins against aggregation to the biological lifetime by which during evolution humans have passed their genes to their progeny. On the basis of these considerations, it is

conceivable to expect that not only the incidence of well-known amyloidoses, but also the number of degenerative diseases one can trace back to the appearance of amyloid aggregates will rise in the near future. Indeed, presently an increasing number of reports demonstrate that the molecular basis of degenerative diseases of previously unknown origin relies on cell damage by aggregates of known proteins (or of proteins that have not yet been identified) previously unsuspected to be deposited in amyloid assemblies (Table 3). For instance, the presence of protein deposits of amyloid type in varying organs and tissues with unknown origin and clinical significance has been described in the basement membrane and lamina propria of the gastrointestinal tract and in the walls of the gastrointestinal blood vessels as well as in the liver [100].

Table 3
Other degenerative diseases with amyloid aggregates

Disease	Aggregated protein	Tissue/cell	Reference
Chronic lung diseases	surfactant protein C	lung	[101,102]
Retinal dystrophies	rhodopsin	retina	[103–106]
Cataract (sporadic/congenital)	γ -crystallins	lens	[107–111]
Familial corneal amyloidosis	lactoferrin	cornea	[112,113]
Inherited corneal dystrophies	β ig-h3	cornea	[114]
Lattice corneal dystrophies	kerato-epithelin	cornea	[115]
Pseudoexfoliation syndrome	?	aqueous humor	[116]
Heredo-oto-ophthalmo-encephalopathy	?	retinal vessels	[117]
Oculopharyngeal muscular dystrophy	PAPB2	skeletal muscle	[118–120]
Sporadic inclusion body myositis	APP, A β	skeletal muscle	[121,122]
Hypotrichosis simplex of the scalp	comeodesmosin	hair follicle	[123,124]
Atherosclerosis	LDL/ApoB100	vessel walls	[126,127]
FENIB	neuroserpin	cerebral cortex	[131,132]
DFNA9	cochlin	inner ear	[147]
Hirschprung disease	RET	enteric nervous system	[148,149]
Cutaneous lichen amyloidosis	RET	papillary dermis	[151]
Charcot–Marie–Tooth-like diseases	myelin protein 22/0	peripheral nerve tissue	[153,154]
Short-chain acylCoA DH deficiency	SCAD	skeletal muscle, brain	[155]
Ageing pituitary	prolactin	pituitary	[156]
Aortic medial amyloidosis	lactadherin (fragment)	aortic wall	[128]
Cancers	p53	tumoural tissues	[133–142]

5.1. Lung diseases

It is well known that amyloid fibril formation by lung surfactant protein C (SP-C) is the molecular basis of lung diseases such as pulmonary alveolar proteinosis [101]. SP-C contains two fatty acid molecules bound via intrinsically labile thioester bonds; upon fatty acid detaching, SP-C undergoes a conformational change from a monomeric α -helix to polymeric β -sheet-rich fibrillar aggregates very similar to the amyloid fibrils. The higher aggregability of fatty acid-free SP-C has been confirmed by *in vitro* experiments [102]. Recently, mutations in the gene encoding SP-C have been shown to be linked to chronic lung disease in children and adults [102] by inducing pro-protein misfolding and toxicity to epithelial cells, with increased BiP transcription, pro-protein trapping in the ER and its rapid degradation via the ubiquitin–proteasome pathway [102].

5.2. Eye tissue diseases

Many degenerative conditions affecting the eye tissues, such as several forms of retinitis pigmentosa, retinal and corneal dystrophies, as well as inherited and sporadic cataract, pseudoexfoliation syndrome and heredo-oto-ophthalmo-encephalopathy have been shown to be associated with the deposition of amyloid aggregates of proteins as diverse as rhodopsin, γ -crystallins, β ig-h3 and others not yet identified.

Diseases affecting the functionality of the retina leading to severe visual impairment and blindness display remarkable genetic and clinical heterogeneity and are a major cause of blindness worldwide. Indeed, over 120 distinct genetic disorders affecting the retina are known, including several forms of retinal degeneration. Recent findings have shown the presence of aggregates of electron-dense rhodopsin both in humans and in animal models of autosomal-dominant retinitis pigmentosa, suggesting a molecular mechanism for disease dominance [103]. At present, over 100 mutations in the rhodopsin gene have been described; many of these mutations destabilize the protein leading to a toxic gain of function by enhancing its tendency to aggregate and leading to cell death [103]. Mutated, misfolded opsin fails to translocate to the plasma membrane and accumulates in the ER and Golgi, where it is found in complex with the molecular chaperones Bip and Grp94 [104]. Mutant rhodopsin does not appear to be efficiently degraded by the proteasome and is ultimately accumulated in intracellular inclusions (aggresomes) together with the wild-type protein and specific opsin-binding proteins even in the absence of proteasome inhibition [103]. Aggresomes contain aggregates of poly-ubiquitinated proteins and proteasome components, whose appearance is thought to result from overwhelming of the normal proteolytic machinery by an excess of misfolded protein [105]. Recent advances have highlighted the key

role performed by specialized opsin chaperones in the molecular pathogenesis of a number of retinal degenerative conditions. Mutation in the chaperones RP2, MKKS and AIPL1 are presently thought to be involved in the appearance of retinal dystrophies such as X-linked retinitis pigmentosa, McKusick–Kaufman syndrome, Biardet–Biedl syndrome and Leber congenital amaurosis (reviewed in Ref. [106]).

Cataract has recently been linked to misfolding and aggregation of specific lens proteins. The eye lens is unique in the organism in retaining all its differentiated post-mitotic cells during the whole life span of an individual. It expresses a peculiar set of specific proteins including γ -crystallins, a family of structural proteins whose mutations are the cause of several autosomal-dominant cataracts in humans [107]. In solution, γ -crystallins are monomeric proteins structurally related to the immunoglobulin protein fold [108]. As IgG and transthyretin, even γ -crystallins are amyloidogenic [109].

Human genetic surveys in families exhibiting juvenile-onset cataract have identified a set of mutations in the gene encoding γ D-crystallin. *In vitro* experiments have shown that the amino acid substitutions in the mutant proteins influence the protein phase transition and solubility, leading to crystallization (as in the aculeiform juvenile-onset cataracts) or *in vitro* aggregation [110]. Other experiments have shown that recombinant mutant γ B-crystallin forms amyloid fibrils under physiological buffer conditions. The data have been confirmed in three different murine cataracts involving mutant γ -crystallins, where large intranuclear inclusions of the protein in the form of filamentous material of amyloid type have been described [111]. Thus it has been proposed that juvenile-onset cataract proceeds through a mechanism involving nuclear targeting and deposition of amyloid-like inclusions; mutant recombinant γ -crystallins would initially disrupt sub-nuclear organization and nuclear function (such as transcription) of the lens fiber cells. The disruption of the transcriptional processes is particularly interesting, providing a parallel with the neurodegenerative diseases resulting from deposition of poly(Q) proteins such as huntingtin [111]. Recently, a role for α -crystallin, a protein with chaperone properties, in preventing the formation of cytoplasmic aggregates of mutant γ B-crystallin and other mutated γ -crystallins has been proposed [111]. Based on *in vivo* and *in vitro* data, it has been proposed that γ -crystallins are important even in the appearance of age-onset cataract [109].

For several years, it has been known that a group of hereditary corneal dystrophies are caused by specific missense mutations in the *BIGH3* gene [112] encoding the β ig-h3 protein mainly found in the extracellular matrix of a wide range of developing and mature tissues. Very recently, it has been reported that a subgroup of hereditary corneal dystrophies are determined by some rare mutations in the gene encoding this protein; these are thought to

cause β ig-h3 misfolding and intracellular deposition into amyloid and have been implicated in disease pathogenesis [113]. As the same mutant β ig-h3 does not form amyloid in other tissues in the patients affected by corneal dystrophy, it has been suggested that the protein may require other specific corneal factors to aggregate into the abnormal deposits found in these corneal dystrophies. Other corneal dystrophies are known for which an amyloid basis has been documented. For example, in familial sub-epithelial corneal amyloidoses (also known as gelatinous drop-like corneal dystrophy), the cornea contains amyloid deposits of lactoferrin, both intact and as fragments, possibly originating from the soluble protein in tears [114,115].

Finally, amyloid aggregates of a yet unidentified protein have been found in the aqueous humour of patients with pseudoexfoliation syndrome, supporting the idea that this degenerative condition may be associated with an amyloid of a serum protein [116]. Similar aggregates have also been found in the retina of patients with heredo-oto-ophthalmo encephalopathy [117]. It has recently been reported that even in this case, the visual loss is primarily caused by retinal degeneration comprising extensive accumulation of amyloid material, both diffusely and in the walls of the retinal vessels [117].

5.3. Skeletal muscle diseases

Several degenerative conditions affecting the skeletal muscle have recently been reported to involve protein aggregates. Oculopharyngeal muscular dystrophy is an adult-onset disease characterized by progressive eyelid drooping, swallowing difficulties and proximal limb weakness. The autosomal-dominant form of this disease is caused by a short (GLG)8–13 expansion in the *PABP2* gene encoding the poly(A) binding protein 2 (PAPB2) that binds the nascent poly(A) tails in the nucleus, thus controlling tail length [118]. Mutant PAPB2, containing a small N-terminal polyalanine expansion, is found in the nucleus of the affected cells as filamentous inclusions that are one of the hallmarks of the disease [119]. The inclusions contain poly(A) RNA, ubiquitin and the proteasome components in addition to a form of PAPB2 more resistant to salt extraction than the soluble protein dispersed in the nucleoplasm [119]. A green fluorescence protein construct with a 19–37 polyalanine domain has been found to cause the appearance of intracellular inclusions and cell death [120]. These findings have led to consider this disorder as a new type of triplet-repeat disease, suggesting that polyalanine expansions induce PAPB2 misfolding and aggregation similarly to proteins with pathological poly(Q) expansions. The presence of polyadenylated RNA molecules in the intranuclear inclusions has led to consider these as “mRNA traps” that prevent the transfer of some key mRNAs to the cytoplasm [119].

Sporadic inclusion body myositis, the most common muscle degenerative disease in elderly people, bears similarities to Alzheimer’s disease brain (reviewed in Ref. [121]). The molecular basis of the disease remains substantially unknown; however, very recently a number of reports have shed light on the possible players in its pathogenic cascade. Indeed, the recent identification of several abnormally accumulated proteins within the muscle fibers of sporadic inclusion body myositis patients provides novel and important clues to disease pathogenesis. These accumulated proteins, comprising the amyloid beta precursor protein and its proteolytic amyloid fragments, have been found to misfold and aggregate within the muscle fibers. This feature, combined with, and possibly caused by, the aging of the intracellular environment, could be the key pathogenic event leading to the vacuolar degeneration and atrophy of the muscle fibers that are characteristic of this disease [122].

5.4. Epithelial tissue diseases

Pathological protein aggregates of amyloid type have been found in epithelial tissues. Recently reported findings highlight the possible molecular basis of hypotrichosis simplex of the scalp (HSS), an autosomal-dominant form of isolated alopecia caused by mutations in the *CDSN* gene. *CDSN* encodes corneodesmosin (CDSN), a glycoprotein expressed in the epidermis and in the inner root sheath of hair follicles thought to function as a keratinocyte adhesion molecule [123]. *CDSN* mutations have been suggested to cause the abnormal proteolysis of wild-type CDSN; indeed, truncated forms of CDSN have been shown to aggregate into high molecular weight oligomers in the superficial dermis and the periphery of hair follicles as well as in vitro, leading to suggest that CDSN aggregates are toxic to the hair follicle cells and hence that HSS may be considered as a new protein misfolding disease [124].

Most recently, a missense mutation in the adhesion G domain of laminin 5 causing a mild form of junctional epidermolysis bullosa has been reported in a patient [125]. Even in this case, most of the protein was misfolded and retained within the ER, although modest quantities were secreted and underwent physiological extracellular proteolytic processing thus providing, at least in part, the adhesion function and explaining the mild phenotype [125].

5.5. Atherosclerosis

Very recently, atherosclerosis has been proposed as a new protein misfolding disease [126]. The conformational features of apo-B100 in LDL are affected by any alteration of the water–lipid interface following chemical modifications such as lipid oxidation possibly leading to protein misfolding, as it has been observed in a fraction of

oxidatively modified LDL (LDL-) isolated in vivo (reviewed in Refs. [126,127]). The latter displays apo-B100 misfolding with almost complete loss of α -structure and increase of β -structure. This structural modification leads to the intracellular accumulation of the lipoprotein in high molecular weight insoluble aggregates displaying resistance to proteolysis and cytotoxicity with inhibition of the ubiquitin–proteasome pathway, reduction of lysosome activity and lysosome disruption (reviewed in Ref. [126]). Thus, it has been proposed that atherogenesis might primarily arise from an initial event destabilizing LDL structure and conformation. This would subsequently lead to protein misfolding, aggregation and oxidation, accumulation in the vascular subendothelial space of modified LDL as cytotoxic, pro-inflammatory and protease-resistant aggregates similarly to other well-known protein deposition diseases (reviewed in Ref. [126]).

Another type of localized protein deposition disease involving arterial vessels is the presence of amyloids in the aortic media in elderly people [128]. This form of amyloidosis, very common in subjects above the age of 50, has no well-established clinical significance but is considered to be involved in the age-related loss of elasticity of the vessels. Aortic medial amyloidosis is different from that associated with atheromatous plaques (see above) in that it consists of fibrils formed from a 50-residue fragment (medin) originating from the internal cleavage of the milk fat globule protein lactadherin [128]. It has not been established which stimuli or biochemical modifications determine the accumulation of this peptide fragment nor the possible clinical impact of the aortic medial amyloid deposits.

5.6. Serpinopathies

Recently, new pathologies belonging to the well known protein misfolding diseases referred to as serpinopathies have been described. The deposition of mutant serpin (serine protease inhibitor) as insoluble aggregates in the affected cells is the hallmark of a number of pathological conditions including thrombosis, immune hypersensitivity, angioedema as well as emphysema and liver cirrhosis. These result from conformational changes leading to protein polymerization in the ER, lack of protein secretion and aggregate deposition in the cytoplasm (reviewed in Refs. [129,130]) (see Section 1).

Although they are not classified among the classical amyloidoses and the aggregates do not display the typical cross- β structure of the amyloids, the serpinopathies bear similarities with the amyloid deposition diseases, and the features of protein polymerization underlying these pathologies provide a model for other conformational diseases resulting from aberrant β -linkages. A very recently described new serpin disease is a form of autosomal-dominant dementia known as familial encephalopathy with neuroserpin inclusion bodies (FENIB) [131]. This con-

dition is caused by a mutation of neuroserpin, a serpin specifically found in brain, homologous to another mutation causing α_1 -antitrypsin aggregation, leading to conformational destabilization of neuroserpin; consequently, protein aggregates arise that become sequestered into specific inclusion bodies known as Collins' bodies found in cells of the deeper layer of the cerebral cortex and in the substantia nigra [132]. As with other familial neurodegenerative diseases, the onset and severity of the symptoms of FENIB have been shown to depend on the type of mutation, its effect on the conformational stability of the protein and the magnitude of the intracellular accumulation of the aggregates, demonstrating that intracellular protein aggregation is by itself sufficient to cause neurodegeneration [132].

5.7. Cancer

Many cancers are associated with the abnormal accumulations of aggregates of wild-type and mutant p53, a three-domain protein performing a key role in tumoural suppression. p53 contains structured, partially folded and natively unfolded segments leading to high conformational flexibility [133,134]. It is known that mutations involving mainly the core domain (p53C) are found in over 50% of all human cancers [135,136]. In some cases, an inactive form of wild-type p53, possibly a conformational variant, can be present allowing malignant cells to arise [137,138]. This is the case of tumours such as neuroblastoma, retinoblastoma, breast cancer and colon cancer, where nuclear and cytoplasmic aggregates of this inactive conformational variant of p53 have been described [135,136]. This folding variant displays dominant negative effects being able to drive the active, wild-type protein into an inactive mutant conformation [139]. The nature of the intracellular aggregates of p53 remains unclear; however, it has recently been reported that, under mild denaturing conditions, p53C can be induced to take a mutant-like conformation leading it to assemble into β -sheet rich, toxic fibrillar aggregates [140]. The tetramerization domain of wild-type and mutant p53 has also been shown to reversibly aggregate into amyloid [141]. These data suggest that p53 may be involved in tumoural genesis in two ways: by a loss of the anti-tumour function of the wild-type or mutated inactive protein, and by a gain of function leading it to assemble into aggregates that are able to recruit the functional molecules present in the cell in a way apparently similar to the action of the prion protein [142]. This behaviour has led to hypothesize that tumours could arise in the elderly even in the absence of mutations of p53 simply following a loss of functionality of the ubiquitin–proteasome system favouring the appearance of p53 folding variants nucleating aggregates able to recruit the wild-type molecules similarly to tumour-promoting mutations.

Very recent data have highlighted the amyloidogenic potential of several protein fragments with possible anti-tumoural activity such as endostatin, angiostatin, throm-

bospondin and other angiogenesis inhibitors presently under clinical trials [143,144]. It has been shown that the anti-angiogenic activity of these peptides follows their proteolytic cleavage from the endogenous proteins and is coupled with the ability to trigger tPA-mediated plasminogen activation [145]; such a behaviour appears shared with other amyloids such as A β and prions, together with the evidence that the cross-beta structure is directly toxic to endothelial cells [145]. Furthermore, plasminogen activation seems to favour cell detachment from the extracellular matrix making cells more susceptible to apoptosis [146].

5.8. Other diseases

The increasing number of degenerative conditions for which an amyloid basis is proposed comprises pathologies affecting several other different organs and tissues. For example, the autosomal-dominant hearing loss and vestibular dysfunction disorder DFNA9 is due to five missense mutations in the FCH/LCCL domain of the *COCH* gene encoding cochlin [147]. The mutant protein appears to be secreted, cleaved and glycosylated adequately by the cells, suggesting that the mutations may result in protein aggregation in vivo over a longer time course, as indicated by the late onset and progressive nature of the clinical symptoms [147].

During the last decade, retention in the ER of proteins targeted to plasma membrane carrying missense mutations has emerged as a generic molecular mechanism for several genetic diseases. Among these, Hirschprung disease is a congenital condition characterized by abnormal development of the enteric nervous system caused by mutations in the *RET* gene [148]. *RET* has a relatively high propensity to misfold, and mutations in its extracellular domain affect protein folding followed by alterations of protein processing in the ER (where ubiquitinated, immature forms of *RET* associated with ER chaperones have been found to accumulate) [149] and protein expression at the cell surface [147].

Hirschprung disease has been described as a loss-of-function disease and it is not clear whether it may be considered a true amyloidosis with amyloid aggregates of the mutated misfolded *RET* or if it belongs to the wider group of protein misfolding diseases without amyloid deposition comprising cystic fibrosis, long QT syndrome, familial hypercholesterolemia and oculocutaneous albinism [150]. A very recent report describes the presence of cutaneous lichen amyloidosis with amyloid deposition in the papillary dermis in a few families harbouring *RET* proto-oncogene mutations in codon 634 [151], suggesting the possibility that mutated *RET* may indeed accumulate into amyloid assemblies.

In addition to some corneal dystrophies (see Section 5.2), lactoferrin amyloid has been found in other tissues such as seminal vesicles, a condition common in elderly males

known as localized senile amyloidoses of the seminal vesicles [152] with uncertain clinical significance.

The presence of intracellular aggregates as aggresomes of the peripheral myelin protein-22 and myelin protein zero and of short-chain acyl-CoA dehydrogenase have been reported in familial diseases such as Charcot–Marie–Tooth-related disorders [153,154] and short-chain acyl-CoA dehydrogenase deficiency [155], respectively. In such diseases, these proteins are over-expressed or carry destabilizing mutations leading to folding defects or misfolding. The mutant proteins remain associated for prolonged periods of time with specific chaperones such as BiP, calnexin in the ER or hsp60 in mitochondria. When the proteolytic machineries are impaired or overwhelmed, protein aggregation results and aggregates may exert their pathological effects in different subcellular compartments, although it has not been established whether these aggregates are of amyloid type or amorphous [154,155].

Finally, it has been known for several years that deposits of amyloid type may be found in the pituitary in elderly people [156]. This finding adds to the well-known localized amyloid of human endocrine organs arising from aggregation of hormones such as amylin, atrial natriuretic factor and calcitonin.

6. Concluding remarks

The data reported in the last 5 years on the molecular basis and occurrence of protein aggregation and aggregate toxicity have led to a re-appraisal of the biological and medical significance of this topic as well as of the biological importance of the molecular machineries aimed at hindering the appearance, into cells, of misfolded proteins and their early, toxic aggregates. The scenario that is emerging is that protein misfolding and aggregation into polymeric assemblies, particularly of amyloid type, can be considered a rather generic behaviour of proteins and peptides; this is based on the physicochemical properties of the common polypeptide backbone and its intrinsic property to assemble into intermolecular beta-sheets endowed with high stability and resistance to proteolysis.

This view is further reinforced by the recently reported findings that pre-fibrillar aggregates of protein and peptides are intrinsically cytotoxic regardless of their amino acid sequence, and that their toxicity relies on common conformational features and, at least in most cases, impairs basically the same biochemical parameters (reviewed in Ref. [5]). Should this hypothesis be confirmed by other findings, it might be concluded that proteins, in addition to displaying well-known beneficial behaviours by supporting all biological functions and, ultimately, life itself, also possess a much less known, hidden dark side making all (or most) of them, potentially

toxic to cells. Evolution must therefore have managed proteins in order to exploit all possible benefits from these molecules while keeping under control and reducing as much as possible their generic aggregating and toxic potential.

Indeed, biological evolution has been successful in this task, and the known cases where the dark face of proteins and peptides results in disease are very limited. The latter refers either to situations where specific mutations destroy the adaptive work of evolution (in the case of familial protein deposition diseases) or to conditions where the organism is out of the selective pressure of evolution such as ageing. However, the idea that any minimal perturbing factor may enhance the intrinsic tendency of proteins to aggregate suggests that protein deposition diseases might be much more widespread than presently believed. Indeed, recent data concerning the variability of the human genome sequence, whose exons contain about 60,000 single nucleotide polymorphisms (an average of 1–2 per gene) make this general hypothesis more likely [157]. At least some of these variations could shift the delicate equilibrium between folded and misfolded molecules remarkably increasing the concentration of the latter. It has been calculated that the destabilization of a protein's native state by as few as 2 kcal/mol (as it may be the case of many single-residue mutations) rises the probability of aggregate nucleation by a factor of over 10^5 [158]. The possibility that the risk of acquiring Parkinson's disease is influenced by genetic polymorphisms is presently under investigation [159].

Another possibility could be linked to epigenetics (the complex changes in the genome, such as DNA methylation or histone acetylation, that modulate gene expression while not affecting DNA sequence); epigenetic factors are increasingly believed to play a highly significant role in the development of common diseases such as cancer [160]. Since ageing is accompanied by changes in DNA methylation, at least in some cases it could lead to up-regulating the expression of specific proteins, stimulating their accumulation and aggregation into cells (reviewed in Ref. [161]). For example, analyses of the methylation status of the gene encoding the amyloid precursor protein (APP) in Alzheimer's disease patients suggest a reduced degree of methylation possibly resulting in increased expression of APP thus raising the intracellular levels of the A β peptides [162].

Findings which have appeared mainly in the last couple of years seem to support the idea that a number of degenerative diseases, both sporadic and familial, of unknown molecular origin and many types of cancers may be considered as amyloidoses or amyloid-related diseases. In this review, a number of these pathologies have been discussed. Although this survey may not be exhaustive of all the most recent reports on this topic, it supports the concept that protein deposition diseases are much more widespread than previously believed and may

account for a quite large number of degenerative conditions. Furthermore, recently reported data highlight another aspect of protein aggregation into amyloid assemblies, showing that, in specific biological systems, this is endowed with physiological significance. The examples discussed confirm that, at least in some instances, nature has exploited the natural tendency of proteins and peptides to polymerize into amyloids to perform specific functions, thus showing that something good may arise even from this dark side of the protein world.

A growing body of data indicate that an increasing number of degenerative diseases are associated with the deposition of aggregates of misfolded proteins in the affected tissues and organs. Such information can be of value to develop therapeutic treatments based on the same approaches as those that are presently under investigation for well known degenerative diseases, such as serpinopathies and amyloidoses, where a protein/peptide deposition is well documented.

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