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Amino acid sequence determinants and molecular chaperones in amyloid fibril formation

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ABSTRACT

Amyloid consists of cross-β-sheet fibrils and is associated with about 25 human diseases, including several neurodegenerative diseases, systemic and localized amyloidoses and type II diabetes mellitus. Amyloid-forming proteins differ in structures and sequences, and it is to a large extent unknown what makes them convert from their native conformations into amyloid. In this review, current understanding of amino acid sequence determinants and the effects of molecular chaperones on amyloid formation are discussed. Studies of the nonpolar, transmembrane surfactant protein C (SP-C) have revealed amino acid sequence features that determine its amyloid fibril formation, features that are also found in the amyloid β-peptide in Alzheimer's disease and the prion protein. Moreover, a proprotein chaperone domain ($CTC_{Brichos}$) that prevents amyloid-like aggregation during proSP-C biosynthesis can prevent fibril formation also of other amyloidogenic proteins.

1. Introduction

The focus of this review is on mechanisms that underlie formation of amyloid fibrils and how this process can be modulated by molecular chaperones. Our interest in this topic goes back to the detailed molecular studies of lung surfactant protein C (SP-C), which were initiated in Jörnvall's and Curstedt's laboratories at Karolinska Institutet in the 1980s [1]. SP-C is the most hydrophobic polypeptide so far purified from mammalians, a feature which makes it difficult to handle experimentally. The initial studies defined the structure, as well as post-translational modifications of SP-C [2], which later on led to the unexpected finding that the native transmembrane SP-C α-helix can convert to amyloid-like β-sheet polymers, and that this is involved in human lung disease [3,4]. NMR spectroscopy and mass spectrometry, in combination with hydrogen/deuterium exchange revealed that the SP-C helix is kinetically stabilized by an unusually high activation barrier for unfolding, and that the life-time of the soluble state equals that of the helical state, i.e. in contrast to the situation for most proteins so far studied, the SP-C α-helix does not unfold and refold continuously [3].

Insufficient amounts of lung surfactant underlie development of respiratory distress syndrome (RDS) in premature infants, a disease that nowadays can be effectively treated by instillation of natural-derived surfactant preparations. The above findings on SP-C metastability suggested an explanation to the aggregation problems encountered earlier when attempting to synthesize SP-C for formulation of an artificial surfactant preparation for treatment of RDS. To circumvent this problem all valine residues in SP-C (which have high β-strand propensity) were replaced with leucine (with high α-helix propensity). This yielded an SP-C analogue with a thermodynamically stable helix that refolds after unfolding, and which can be synthesized in large amounts without aggregation problems. This analogue mixed with synthetic phospholipids can be used to treat RDS in animal models [5].

The inability of the SP-C α-helix to refold after unfolding implies that its formation during SP-C biosynthesis does not occur spontaneously but requires other cellular factors. We have recently found that the C-terminal domain of proSP-C, $CTC_{Brichos}$, which is localized in the ER lumen, works as a chaperone that binds unfolded (pro)SP-C and thereby prevents it from aggregation [6]. Intriguingly, mutations that are associated with human lung fibrosis are to a large extent localized to this chaperone domain. Data from cell models show that these mutations inactivate the chaperone function and cause SP-C misfolding and aggregation, suggesting that mutations in $CTC_{Brichos}$ may cause protein misfolding *via* an indirect mechanism. We speculate that lung fibrosis associated with mutations in $CTC_{Brichos}$ is associated with amyloid formation, a possibility that currently is under investigation.

Abbreviations: Aβ, amyloid β-peptide; Hsp, heat-shock protein; RDS, respiratory distress syndrome; SP-C, surfactant protein C.

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2. Properties of amyloid and associated diseases

A number of diseases are linked to the misfolding and subsequent aggregation of proteins. In some of these disorders the protein self-associates into aggregates called amyloid fibrils (Fig. 1). Today, around 25 diseases are associated with amyloid, including e.g. Alzheimer's disease and Creutzfeldt–Jakob's disease. Amyloid fibrils are stable, insoluble aggregates composed of β-sheets with their strands running perpendicular to the axis of the fibril. In order to be classified as amyloid certain criteria must be met. Depositions must occur *in vivo*, the fibrils formed should be straight, unbranched and show a typical cross-β X-ray diffraction pattern (signals at 4.7 and at 10–11 Å) [7], and in addition staining with the dye Congo red should result in green birefringence in polarized light. The process of fibril formation is a nucleation dependent process, characterized by a critical concentration below which no aggregation will occur, a lag-phase during which a nucleus (oligomer) is formed, followed by an elongation phase and finally a steady state phase. In addition to the classical amyloid, it has recently been suggested that also functional protein assemblies have amyloid-like properties, and they are consequently referred to as functional amyloid [8,9]. Functional amyloid generates bacterial pili and human pigment binding templates, or regulates translation in yeast. It was recently found that several peptide hormones can be stored in an amyloid state in secretory granules, and this might be regarded as a type of functional amyloid [9].

It is not established how amyloid gives rise to disease. In some cases, e.g. in lysozyme amylodosis, huge amounts of amyloid fibrils can accumulate and this is likely to cause disturbed cellular homeostasis. In line with this, the classical amyloid cascade hypothesis states that Alzheimer's disease is caused by fibril and plaque formation of Aβ in the central nervous system. However, there is no correlation between plaque load and disease progression, and more recently the hypothesis has been modified to include Aβ assemblies of sizes intermediate to monomeric and fibrillar forms, which today are considered to be the main source of cytotoxicity [10].

3. Amino acid sequence determinants of amyloid fibril formation

It has long been a matter of discussion whether the amyloidogenic potential of a protein or peptide can be assessed based on its amino acid sequence. Algorithms that combine polarity, α-helix and β-strand propensities, charge, and side-chain hydrogen bonding properties of the constituent amino acid residues can predict relative protein aggregation rates and identify regions prone to form β-sheet aggregates [11,12]. However, no obvious sequence homology has been described for disease-associated amyloid proteins, and it has been suggested that the ability to form amyloid fibrils is a general property of the polypeptide backbone [13]. The observation that certain proteins are much more prone to aggregation than otherwise similar proteins, and the fact that only a very minor subset of all proteins form amyloid *in vivo*, however, suggest the existence of sequence-specific elements that facilitate aggregation of the polypeptide into amyloid fibrils. Another possibility is that sequence determinants for amyloid are generally occurring, but that they are able to promote amyloid fibril formation only under certain circumstances, e.g. high enough concentration and conditions that de-stabilize the native fold. Synthetic peptides are a powerful tool to investigate the interplay between a peptide's amino acid sequence and its ability to form amyloid fibrils. The use of peptide models also provides comparatively simple systems for the study of inhibition and reversion of fibril formation. Studies using fragments of amyloidogenic proteins that can self-assemble into fibrils have shown that short sequences are likely to govern amyloid formation. One such example is the KLVFFAED motif, a known amyloidogenic sequence of the amyloid β-peptide (Aβ), which contains positively and negatively charged residues and has a high β-strand propensity. Tjernberg and co-workers showed that it is possible to design synthetic amyloidogenic tetrapeptides by retaining these characteristics [14]. It was also found that rather minor alterations in sequence, such as replacement of valine or phenylalanine with leucine or alanine, can abolish fibril formation and that the amyloidogenic properties of short peptides can be abolished by introduction of adjacent sequence motifs such as β-turns [15]. These data indicate that both the amino acid sequence as such and its structural context affect the ability to form amyloid fibrils.

It has been suggested that the occurrence of certain amino acid residues is a key feature of amyloid fibril-forming ability. Gazit has identified the stacking of π-bonds as an important feature in amyloid peptide assembly [16]. Analysis of the amyloidogenic regions in fibril-forming proteins showed a high occurrence of the aromatic residues phenylalanine and tyrosine, which have a high propensity to stack the delocalized π-electron rings in a parallel manner. Studies with short peptides have confirmed that even two consecutive phenylalanine residues are sufficient to facilitate assembly into nanotube-like structures [17], and stacks of aromatic residues were observed in crystal structures of several

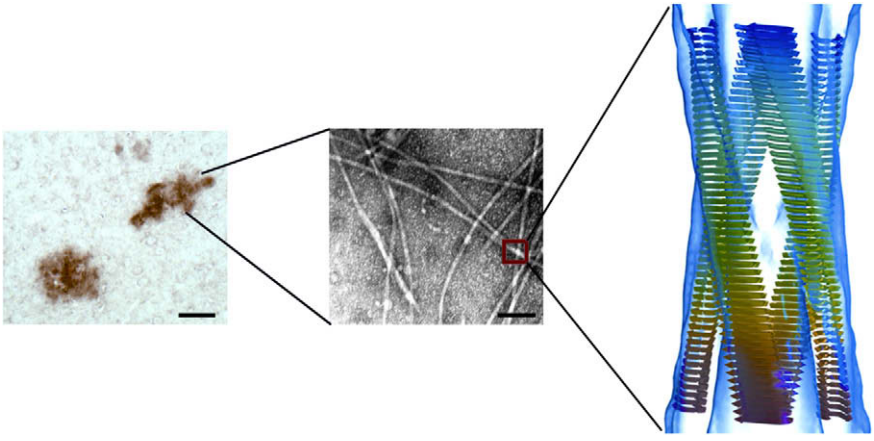

Fig. 1. Structural organization of amyloid and its fibrils. Amyloid plaques (left), fibrils (middle) and cross β-sheet polymers (right). Scale bars represent 50 μm in the plaque image and 100 nm in the fibril electromicrograph. The micrograph of amyloid plaques was kindly provided by Siwei Peng.

amyloidogenic peptides [18]. However, the possibility to design non-aromatic peptides that assemble into amyloid fibrils suggests that aromatic residues may not be an essential component for fibril formation.

Another approach to the use of peptide models was presented by Nelson et al. in which microcrystals formed by short synthetic peptide segments from the yeast Sup35 prion were used for structure determination [19]. It was suggested that the microcrystalline packing of short peptides closely resembles the packing of the amyloidogenic segments in amyloid fibrils, and based on the structural data, the arrangement of side-chains into closely packed steric zippers was identified as another important feature of amyloid formation. Yeast prions, like Sup35, belong to the class of functional amyloid. Since disease-associated amyloid differ from functional amyloid in several aspects, it is possible that their sequence determinants differ. Aβ fibrils, for example, do not show steric zipper packing [20]. Sawayan et al. have extended the microcrystal approach to amyloidogenic segments from a variety of amyloid-forming proteins and confirmed the presence of steric zipper structures in 11 fibril-forming peptides [18].

Lopez da la Paz et al. have developed a computer-based method for *de novo* design of amyloid peptides based on the ability of a short amino acid sequence to stabilize or de-stabilize a β-sheet structure [21]. A different approach to prediction was taken by Kallberg and co-workers [22]. They reported that, while no specific amyloidogenic signature could be assigned in protein sequences, the ability to form amyloid fibrils could be controlled by polypeptide segments that adopt helical conformations, although they have high β-sheet propensities. SP-C is α-helix/β-sheet discordant, i.e. its helix is built from a stretch of residues that has a very high intrinsic tendency to form a β-strand. This not only affords a likely explanation to the tendency of SP-C to convert from α-helix to β-sheet fibrils, but intriguingly, α-helix/β-sheet discordance was found in Aβ associated with Alzheimer's disease and the prion protein underlying spongiform encephalopathy. Such discordant helices contain a high number of amino acids that are overrepresented in β-strands. Such "frustrated" helical segments are particularly prone to undergo α-helix → β-strand conversion and may serve as a trigger for amyloid fibril formation by native proteins. In contrast to the situation for mature SP-C, the α-helix of a processing intermediate on the pathway from proSP-C to SP-C was found be long-term stable and not unfold to form β-sheet aggregates [23]. The increased stability of this intermediate is mediated by the presence of a propeptide segment that may interact with the N-terminal part of the α-helix and thereby increase the energy barrier for unfolding. Hammarström et al. demonstrated how conversion of the native transthyretin structure into β-sheet fibrils can be suppressed by trans-stabilization [24]. The hypothesis that stabilization of the native state is a means to prevent amyloid fibril formation is further supported by our recent findings that designed low-molecular mass ligands, which stabilize the helical form of Aβ, prevent fibril formation and reduce Aβ toxicity in cell models and in transgenic flies that over-express Aβ in the CNS [25].

4. General and specific chaperones in amyloid prevention

The proprotein of SP-C, proSP-C, contains a C-teminal part, called CTC, which harbours a Brichos domain. The name of the domain is derived from its existence in the *Bri* protein, *cho*ndromodulin, and *S*P-C, but it is present also in other proteins associated with degenerative and proliferative disorders. Several functions were initially proposed for it, including a chaperone function [26]. $CTC_{Brichos}$ has been shown to be important for correct folding of proSP-C in the ER and to be able to prevent SP-C amyloid fibril formation *in vitro* [6]. In addition, this anti-amyloid chaperone activity extends to other amyloidogenic proteins such as Aβ and medin which forms amyloid in the aortic wall [27]. Studies of the mechanism behind this chaperone-like action have shown that $CTC_{Brichos}$ has high binding affinity for stretches of the amino acid residues isoleucine, valine, phenylalanine and leucine, all of which promote membrane insertion according to the biological hydrophobicity scale [28]. $CTC_{Brichos}$ is also able to distinguish whether its target peptide is in helical and non-helical conformation, binding only in the latter case. These findings suggest that $CTC_{Brichos}$ is the first known chaperone that combines the recognition of "unfolded transmembrane segments", i.e. non-helical segments rich in isoleucine, valine, leucine or phenylalanine, with the prevention of amyloid fibril formation. The importance of the Brichos domain for the binding to aggregation-prone protein segments is further highlighted by the fact that another Brichos domain, evolutionarily quite distant from $CTC_{Brichos}$, binds to a segment of its proprotein that has high β-strand propensity, and can also prevent the aggregation of $A\beta_{1-40}$ *in vitro* [29].

The idea that chaperones may have anti-amyloid properties has been proposed earlier and numerous studies have been conducted to elucidate the potential role of molecular chaperones in the formation of amyloid (Table 1). Molecular chaperones are one of several systems that have evolved to assist folding in the cell and to counter misfolding to occur. Chaperones bind to proteins and aid in the folding process but they can also recognize misfolded proteins and target them for degradation. In the case of amyloid prevention a number of intracellular heat-shock proteins (Hsp) have been studied. For example, Hsp70 and Hsp90 have been shown to affect fibril formation of $A\beta_{1-42}$ at an early stage [30–32] and moreover Hsp70 also affects the early stages of aggregation of α-synuclein [33]. Hsp104 has been shown to interact with various pre-fibrillar species of $A\beta_{1-42}$ and thereby suppress the formation of fibrils [34]. In addition, Hsp104 is able to disaggregate Sup35-fibrils *in vitro* [35]. Small heat-shock proteins (sHsp) also make up a family of intracellular molecular chaperones that possesses anti-amyloid properties. sHsp have been found to co-localize with for example senile plaques associated with Alzheimer's disease and to inhibit Aβ fibril formation [36]. It should be pointed out that many proteins have been found in amyloid plaques, probably as an effect of their "sticky" nature, and therefore co-localization alone is not necessarily an indication of disease-related interactions. Part of the α-crystallin domain of the sHsp αA-crystallin has also been shown to inhibit fibril formation of Aβ, indicating a potential preventive role [37]. Most of the disease-related amyloid deposits are found extracellularly. In addition to the intracellular chaperones discussed above evidence of an extracellular quality control system has been reported [38]. This system so far includes the extracellular proteins clusterin, haptoglobulin and α_2-macroglobulin [39–41]. These proteins are upregulated during stress and are able to protect a range of proteins from stress-induced aggregation. In addition, they have been found in association with amyloid deposits, to interact with pre-fibrillar species and to inhibit amyloid formation [42,43]. It is proposed that these chaperones recognize misfolded or aggregated proteins and promote their uptake into nearby cells for further degradation [44].

The importance of chaperones in the prevention of fibril formation is becoming more obvious and elucidating their mode of action could provide a novel route in the search for anti-amyloid therapies. However, the effect of inhibition observed varies between different chaperones and also with the ratios used, indicating a complex mechanism. In addition, chaperones are by nature designed not to bind their targets too strongly, which potentially poses a problem since it could result in the accumulation of misfolded and aggregated species. It is further not known if the observed up-regulation of chaperones and/or their accumulation at aggregation sites represent a protective mechanism or is part of

Table 1
Selection of known molecular chaperones and examples of their effect on amyloid fibril formation. EC, extracellular chaperone; Hsp, heat-shock protein; sHsp, small heat-shock protein.

Chaperone	Class	Inhibits/alters aggregation of	Reference
Hsp70	Hsp	$A\beta_{1-42}$, α-synuclein	[32,33]
Hsp90	Hsp	$A\beta_{1-42}$	[32]
Hsp104	Hsp	$A\beta_{1-42}$, Sup35	[34,35]
Hsp 27	sHsp	$A\beta_{1-40}$	[36,45]
αB-crystallin	sHsp	$A\beta_{1-40}$, $A\beta_{1-42}$, β_2-microglobulin	[46]
Clusterin	EC	$A\beta_{1-42}$, calcitonin, lysozyme, SH3, α-synuclein, β_2-microglobulin, κ-casein	[43]
Haptoglobulin	EC	$A\beta_{1-42}$, calcitonin, lysozyme	[42]
α_2-Macroglobulin	EC	$A\beta_{1-42}$, calcitonin, lysozyme	[42]
$CTC_{Brichos}$	Brichos	SP-C, $A\beta_{1-40}$, medin	[6,27]

the disease process. This needs to be clarified in order to take advantage of this natural protective system. In spite of these obstacles, finding new types of chaperone-like molecules with specified targets may be a promising new therapeutic strategy.

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