
REVIEW

Amyloid accomplices and enforcers

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Abstract

Amyloid-related diseases are often ascribed to protein “misfolding.” Yet in the absence of high-resolution structures for mature fibrils or intermediates, the connection between the mechanism of amyloid formation and protein folding remains tenuous. The simplistic view of amyloid fibrillogenesis as a homogeneous self-assembly process is being increasingly challenged by observations that amyloids interact with a variety of cofactors including metals, glycosaminoglycans, glycoproteins such as serum amyloid P and apolipoprotein E, and constituents of basement membranes such as perlecan, laminin, and agrin. These “pathological chaperones” have effects that range from mediating the rate of amyloid fibril formation to increasing the stability of amyloid deposits, and may contribute to amyloid toxicity. An increasing appreciation of the role of accessory molecules in amyloid etiology has paved the way to novel diagnostics and therapeutic strategies.

Keywords: Alzheimer A β peptide; protein folding; extracellular matrix proteins; heparan sulfate proteoglycans; basal lamina

According to the “Thermodynamic Hypothesis,” the functional native structure of a protein is determined by the full complement of interactions formed by the protein’s amino acid sequence in a given environment (Anfinsen 1973). Whereas in complementary duplex DNA one strand guides the structure of the other, all of the information for protein folding is contained within the protein sequence. In vivo, chaperones indirectly enhance the efficiency of protein folding by disentangling proteins trapped in aggregative side reactions but do not affect the structure or properties of the final native state (Dobson 2003). This has been demonstrated in among other experiments by the correct folding of proteins prepared by solid-phase methods in vitro (Anfinsen

1973; Dawson and Kent 2000). While the properties and structures of functional native proteins are encoded by their amino acid sequences (Anfinsen 1973), there is increasing evidence that the properties of amyloid deposits formed in vivo depend strongly on a variety of cofactors (Table 1). As such, the mechanism of amyloid formation may be more a consequence of improper heterologous interactions than improper protein folding.

A large variety of proteins and peptides can self-assemble into aggregates that share tinctorial and morphological hallmarks of the amyloid fibrils found in situ (Rochet and Lansbury 2000). These include bacterial proteins (Alexandrescu and Rathgeb-Szabo 1999), proteins with an α -helical native structure such as myoglobin (Fandrich et al. 2001), and designed peptides as small as six amino acids (Lopez De La Paz et al. 2002). Inasmuch as polypeptides with unrelated sequences and functions can be driven to form fibrils in vitro (albeit under conditions removed from those for which the native state has been selected), it has been hypothesized that amyloid formation is a general property of proteins (Dobson 1999). Yet the amyloids associated with human disease are formed by only ~20 different polypeptide precursors (Coker et al. 2000). While much evidence suggests a correlation between protein stability and propensity for

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Abbreviations: A β , Alzheimer β -peptide; AD, Alzheimer’s disease; apoE, apolipoprotein E; APP, (Alzheimer) amyloid precursor protein; IAPP, islet amyloid polypeptide; GAG, glycosaminoglycan; HSPG, heparan sulfate proteoglycan; LNS, laminin neurexin and SHBG-like domain; PrP^C, soluble form of prion protein; PrP^{Sc}, protease-resistant form of prion protein; SAA, serum amyloid A; SAP, serum amyloid P component.

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amyloid formation (Chiti et al. 2000), proteins implicated in amyloid pathology such as lysozyme, transthyretin, and β -microglobulin, do not seem remarkably unstable in light of estimates that as much of 40% to 60% of eukaryotic proteomes could be intrinsically disordered (Dunker and Obradovic 2001). A propensity to form partially folded conformations is also unlikely to be a hallmark of amyloidogenic proteins (Booth et al. 1997) given the proportion of large, multidomain proteins in the human proteome. Moreover, any protection afforded by intracellular chaperones against fibril deposition must precede the typical extracellular deposition of amyloids (Hirschfield and Hawkins 2003). With the exception of systemic amyloidosis where deposits occur in all tissues except the brain, amyloid deposits are focal; that is, amassed in specific locations (Sipe 1992; Bush 2002; Hirschfield and Hawkins 2003). Alzheimer's β -amyloid ($A\beta$) is deposited in synapses and the basement membranes of brain blood vessels, in spite of the fact that $A\beta$ is expressed in a variety of tissues (Cotman et al. 2000; Bush 2002). Deposits of hemodialysis-related β 2-microglobulin amyloids are preferentially distributed in joints and connective tissues, and more rarely in visceral organs (Ohashi et al. 2002). Amyloids derived from islet amyloid polypeptide (IAPP) are deposited in the pancreatic islets of Langerhans. Deposition rarely occurs in persons without type II diabetes, although IAPP is expressed in normal individuals at levels manyfold higher than those required to form amyloid fibrils in vitro (Jaikaran et al. 2003; Potter-Perigo et al. 2003; Larson and Miranker 2004). Taken together, these observations challenge the view that amyloids result from protein misfolding, and suggest a prominent role for extrinsic cofactors in the etiology of amyloid-linked pathologies.

Although amyloid deposits are often described as inert, there is evidence that amyloid formation can be reversible,

at least in some cases. Radioactively labeled serum amyloid P component (SAP) a cofactor present in all amyloid deposits formed in vivo, has been used to demonstrate dynamic turnover of fibrils and regression of deposits after the supply of amyloid precursor protein is removed (Pepys 2001; Pepys et al. 2002). In an elegant study, competition experiments involving ^{125}I -labeled fibril precursors were used to demonstrate the reversibility of $A\beta$ peptide deposition on plaques from Alzheimer's disease (AD) brain tissue (Maggio et al. 1992). Reversible binding was attributed to a steady-state equilibrium between the $A\beta$ peptide in amyloid deposits, and free in solution (Maggio et al. 1992). Fluorescence energy transfer between $A\beta$ peptide analogs carrying either a tryptophan or AEDANS probe was used to demonstrate the reversibility of $A\beta$ fibril formation, and to obtain kinetic rate constants for exchange between $A\beta$ monomers and aggregates (Huang et al. 1997). Similarly, when model fibrils formed by the *Escherichia coli* protein CspA were incubated in D_2O , all sites in the protein gave uniform hydrogen exchange rates; an observation most consistent with exchange occurring through dissociation of CspA from the fibrils (Alexandrescu 2001). Cofactors in amyloid diseases may shift the equilibrium between soluble and insoluble forms of the amyloidogenic proteins. Thus, depletion of metal ions by chelators facilitates aqueous dissolution of $A\beta$ deposits isolated from postmortem AD brains (Cherny et al. 1999). Other types of cofactors appear to nucleate amyloids or have roles in amyloid assembly (Castillo et al. 1999; Cohlberg et al. 2002; Ancsin 2003; Deleault et al. 2003).

Accessory molecules in amyloid formation

Metals

The transition metals zinc, copper, and iron have been implicated in various aspects of Alzheimer's disease (Bush

Table 1. Summary of principal amyloid cofactors and their effects

Cofactor	Effect ^a	Reference
Zn ²⁺	Enhances $A\beta$ aggregation	Bush et al. 1994
Cu ²⁺ , Fe ³⁺	Increase $A\beta$ toxicity via generation of free radicals	Bush 2003
RNA	Converts PrP ^C prion to protease resistant form	Deleault et al. 2003
Cholesterol	Metabolites may facilitate $A\beta$ fibril nucleation	Zhang et al. 2004
GAG	Promotes fibrillogenesis, stabilizes fibrils	Ancsin 2003
Insulin	Inhibits IAPP fibril growth	Larson and Miranker 2004
ApoE	Affects brain $A\beta$ concentration, promotes fibrils	Bales et al. 2002
ApoJ	Increases $A\beta$ toxicity	DeMattos et al. 2002b
SAP	Stabilizes amyloid	Pepys 2001
Aggrin	Promotes $A\beta$ fibrillogenesis, stabilizes fibrils	Cotman et al. 2000
Perlecan	Promotes fibrillogenesis, stabilizes fibrils	Castillo et al. 1999
Laminin	Inhibits $A\beta$ fibrillogenesis	Castillo et al. 2000

^a Where the specificities of cofactors for different types of amyloids are not given, the effects are thought to extend to all types of amyloid. For example, SAP appears to stabilize all known types of amyloid. In cases where specificities are given, the cofactors may play roles in additional processes. For example, Cu²⁺ may be involved in prion pathology and Parkinson's disease in addition to its role in Alzheimer's.

and Tanzi 2002; Bush 2003). Physiological concentrations of Zn^{2+} (μM) are sufficient to convert soluble A β peptide into protease-resistant amyloid-like aggregates (Bush et al. 1994; Bush 2003). Zinc-induced aggregation involves residue His13 of the A β peptide (Liu et al. 1999), which participates in His13 (N ϵ 1)– Zn^{2+} –His 13 (N ϵ 1) cross-links between A β monomers (Miura et al. 2000). In contrast to the human peptide, rat A β , which has a His13 substituted by arginine, is not precipitated by physiological zinc concentrations (Bush et al. 1994). Extracellular zinc concentrations reach as high as ~ 0.3 mM during synaptic transmission but are subsequently reduced by an energy-dependent zinc-uptake transporter to levels below 0.5 μM (Bush and Tanzi 2002; Lee et al. 2002; Bush 2003). Transport into synaptic vesicles requires the zinc transporter protein ZnT3. Mice bearing cerebral amyloid plaque pathology but lacking the ZnT3 gene show a marked reduction in A β deposits, and an increase in soluble A β (Lee et al. 2002).

While zinc is the only metal that can precipitate A β at physiological concentrations, copper and iron have more direct roles in A β toxicity (Bush 2003). Copper is found in synaptic vesicles at concentrations an order of magnitude lower than zinc, but Cu^{2+} binds A β (1–42) with 10^{-18} M affinity (Bush and Tanzi 2002). Because the affinity is so high, it is likely that the physiological form of A β is Cu^{2+} -bound (Bush 2003). The A β peptide catalyzes hydrogen peroxide (H_2O_2) formation through the reduction of Cu^{2+} and Fe^{3+} , using O_2 and biological reducing agents as substrates (Bush 2003). Hydroxyl radicals are also produced in the reaction, and attack the susceptible A β peptide (Huang et al. 1999) leading to the formation of SDS-resistant A β oligomers with enhanced neurotoxicity (Walsh et al. 2002; Bush 2003). These oligomers may further seed zinc-induced amyloid deposition (Bush 2003). Copper and iron levels rise in senescence, and both metals are found in high concentrations in AD plaques (Bush 2003). The overexpression of amyloid precursor protein (APP), and increases in A β peptide levels, may themselves be responses to an increased concentrations of metal ions (Rogers et al. 2002). Although the precise biological function of APP remains unknown, the presence of Zn^{2+} and Cu^{2+} binding sites in the N-terminal and A β domains of the protein suggest a role in metal homeostasis (Maynard et al. 2002; Barnham et al. 2003).

In addition to AD, metal ions have been implicated in a number of other amyloid pathologies. Binding of Cu^{2+} to β -microglobulin is a contributing factor in hemodialysis-related amyloidosis (Morgan et al. 2001). Fibril formation by α -synuclein is accelerated by several metal ions (Al^{3+} , Cu^{2+} , Fe^{3+}) implicated as risk factors for Parkinson's disease (Uversky et al. 2001). The prion protein (PrP) binds copper and zinc and like APP may have a role in metal homeostasis (Watt and Hooper 2003). Removal of metal binding sites simultaneously affects the neurotoxicity and the ability of a 106–126 peptide fragment of human prion

protein to form fibrils (Jobling et al. 2001). Binding of Cu^{2+} and Zn^{2+} have been suggested to enable interconversion between different subtypes of the infective PrP^{Sc} prion (Wadsworth et al. 1999).

Side-products of cholesterol metabolism

Derivatives of cholesterol containing aldehyde groups are generated as a result of antibody-catalyzed ozone production during the inflammation processes associated with atherosclerosis (Zhang et al. 2004). It was recently shown that some of the ozonolysis products of cholesterol markedly enhance the assembly of A β peptides into fibrils (Zhang et al. 2004). The aldehyde groups from the cholesterol metabolites can condense with amine groups on A β peptides. The resulting covalently modified A β peptides are more hydrophobic, and appear to form exceptionally potent nuclei for fibril formation, thereby accelerating fibrillogenesis (Zhang et al. 2004).

IAPP and insulin

Fibrils of islet amyloid polypeptide (IAPP) form at peptide concentrations 100-fold lower than those found in the secretory granules of the pancreatic islet β -cells, where IAPP is made. This raises the question of how fibril formation is prevented in normal individuals. Recent reports indicate that insulin, stored together with IAPP in secretory cells, inhibits IAPP amyloid formation (Jaikaran et al. 2003; Larson and Miranker 2004). Insulin binds to the surface of IAPP fibrils without causing their disassembly (Jaikaran et al. 2003; Larson and Miranker 2004). Binding of insulin appears to inhibit IAPP fibril elongation and may prevent aggregation of fibers into higher order arrays (Larson and Miranker 2004).

Prion conversion by RNA

The central event in prion pathology is the conversion of normal PrP^C to a pathological protease-resistant form of the protein PrP^{Sc}. This conformational switch occurs ineffectively in vitro, requiring a large 50-fold excess of the protease-resistant form of the prion protein (Deleault et al. 2003). Recent results show that the conversion to protease resistant prion reaches stoichiometric efficiency in the presence of RNA (Deleault et al. 2003). Conversion is stimulated by mammalian but not by invertebrate RNA, suggesting that RNA is a host-specific cofactor (Deleault et al. 2003). Conversion to the protease resistant form of the prion protein is also stimulated by heparan sulfate, and is inhibited by thiol blockers (Supattapone 2004).

Glycosaminoglycans

Together with collagens, glycosaminoglycans (GAGs) and proteoglycans are the principal components of the extracellular matrix—the connective meshwork that contrib-

utes to the stability, development, communication, and migration of cells (Bosman and Stamenkovic 2003). The extracellular matrix includes specialized structures such as basement membranes to which cells adhere. Amyloid deposits are often localized to these structures, and often contain tightly bound components of basement membranes (Sipe 1992; Fukuchi et al. 1998; Ancsin 2003; Hirschfield and Hawkins 2003).

The GAGs are a group of negatively charged heterogeneous polysaccharides built up from repeating disaccharide units (Fig. 1). Proteoglycans are formed when GAG chains are covalently attached to the serine residues of various core proteins. Compared to glycoproteins, proteoglycans are much larger and have a higher proportion of polysaccharide (up to 95% by weight). Proteoglycans often have multiple GAG chains attached; these are unbranched, and have from 10 to more than 100 carbohydrate residues. Glycosaminoglycans and proteoglycans are found associated with all types of amyloid deposits (Sipe 1992; Fukuchi et al. 1998; Ancsin 2003; Hirschfield and Hawkins 2003), including the neurofibrillary tangles formed by the microtubule-associated protein tau, which are specific to Alzheimer's disease (Fukuchi et al. 1998; Cohlberg et al. 2002). Of the seven different types of GAGs (Fig. 1), heparan sulfate and dermatan sulfate are the most prominent in amyloid deposits (Hirschfield and Hawkins 2003). Interestingly, GAGs also associate with deposits of α -synuclein in vitro (Cohlberg et al. 2002). The protein α -synuclein is the primary component of the Lewy bodies observed in the substantia nigra of patients with Parkinson's disease (Cohlberg et al. 2002). In contrast to extracellular amyloids, α -synuclein deposits are intracellular.

Proteoglycans and GAGs have been implicated in the nucleation of fibrils. They can also stabilize mature fibrils against dissociation (Yamaguchi et al. 2003) and proteolytic degradation (Gupta-Bansal et al. 1995). Heparan sulfate and HSPGs have been shown to bind to a number of amyloid precursor proteins including β 2-microglobulin (Ohashi et al. 2002) and Alzheimer's APP. Recent progress has been made in identifying HSPG binding sites in Alzheimer's amyloid precursor protein (APP) by X-ray crystallography (Rossjohn et al. 1999; Wang and Ha 2004). Heparan sulfate and HSPGs also bind to the fibrillar forms of amyloid-forming proteins, typically with much stronger affinity than to the monomeric forms. They have been shown to facilitate the formation of fibrils of IAPP (Castillo et al. 1998; Potter-Perigo et al. 2003), A β (Castillo et al. 1999), SAA (Ancsin and Kisilevsky 1999), tau protein (Goedert et al. 1996), α -synuclein (Cohlberg et al. 2002), and prion protein (Supattapone 2004). It has been proposed that GAGs may have a scaffolding role, promoting fibrilization-prone conformations of the amyloid precursor proteins (Cohlberg et al. 2002; Ancsin 2003). Although the morphologies and tinctorial properties of amyloids from a variety of

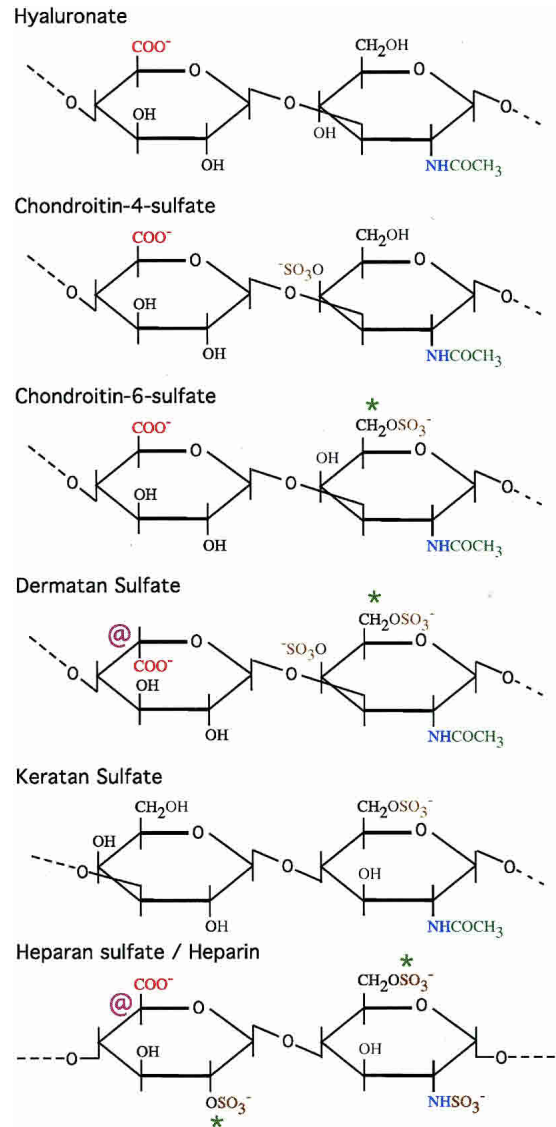


Figure 1. Repeating disaccharide units found in different types of glycosaminoglycans. The disaccharide units consist of a hexuronic acid such as gluconate or iduronate, and a hexosamine such as N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc) (Garret and Grisham 1999; Ancsin 2003). The symbol “@” indicates that the carboxyl group on the 5-carbon can exist in multiple epimerization states. The symbol “*” indicates that the substituent can be either a hydrogen (—H) or a sulfate group (—SO₃⁻). Negatively charged groups are shown in red (—COO⁻) and brown (—SO₃⁻). After synthesis, GAGs are extensively processed in the Golgi by enzymes that catalyze their sulfonation and epimerization. Hyaluronate is the only GAG that is not sulfated, and the only GAG that does not occur in proteoglycans. Heparin and heparan sulfate are shown together. Heparan sulfate is less extensively modified than heparin, has fewer sulfate groups, is less negatively charged, and is more difficult to obtain than heparin. Heparin occurs mainly in the cytoplasmic granules of mast cells, while heparan sulfate is found on cell surfaces and in the extracellular matrix.

sources appear uniform (Dobson 1999, 2002), EM studies suggest differences between fibrils formed in vitro and in situ. In EM images of deposits isolated from patients with

amyloid disease, proteoglycans, and SAP appear to form the cores of the structures, whereas the specific “misfolded” protein characteristic of the amyloid type is found on the surface of the fibrils (Inoue et al. 1997; Inoue and Kisilevsky 1999).

GAG chains bind amyloid fibrils noncovalently, and with high affinity. Complexes of GAGs with A β (1–40) fibrils, for example, are sufficiently stable to protect the glycan chains from digestion with the enzyme heparanase (Bame et al. 1997). This raises the mechanistic question of how the polar and flexible GAG polyanions come to associate with the presumably hydrophobic and rigid interior of amyloid fibrils. Heparin binds to A β , IAPP, and α -synuclein, with a much higher affinity for the fibril than for the soluble states. These observations indicate that recognition depends on the quaternary structure of the aggregates (Watson et al. 1997; Cohlberg et al. 2002). Binding of GAGs to A β and to α -synuclein fibrils is strongly correlated with sulfate content, and hence, with the number of negative charges on the glycans (Castillo et al. 1999; Cohlberg et al. 2002). Chemically desulfated heparin fails to enhance A β fibril formation (Castillo et al. 1999). Oddly, it has been shown that α -synuclein fibril formation can be stimulated both by negatively charged GAGs and by positively charged poly-lysine (Cohlberg et al. 2002).

Heparin and heparan sulfate bind to a number of native proteins through basic amino acid clusters. Some of these proteins contain consensus sequence motifs of the type XBBXB and XBBBXXBBX, where B are the basic residues Arg, Lys, His (Cardin and Weintraub 1989; Ancsin and Kisilevsky 1999; Cohlberg et al. 2002). Other heparin-binding proteins lack consensus sequence motifs, and recognize epitopes defined by the arrangements of basic residues in the protein’s three-dimensional structure. The lack of sequence conservation between different types of amyloid precursor proteins suggests that GAG-binding epitopes are determined by the organization of basic amino acids in the quaternary structures of amyloid fibrils. In the case of proteoglycans, both the GAG chains and the core protein can potentially contribute to association with amyloid. Experiments with Alzheimer’s A β peptides in vitro suggested that heparan sulfate alone can facilitate the conversion of A β peptides into fibrils (Fraser et al. 1992). By contrast, both heparan sulfate and the core protein of perlecan were needed to form amyloid deposits in vivo (Snow et al. 1994).

Accessory proteins and proteoglycans

SAP

Perhaps the best understood amyloid cofactor is serum amyloid P component (SAP). This glycoprotein is present in all amyloid deposits formed in situ, where it stabilizes fibrils and lowers their susceptibility to proteolytic degradation

(Hind et al. 1984; Emsley et al. 1994; Pepys et al. 1994; Hamazaki 1995; Tennent et al. 1995; Pepys 2001). When found in amyloid, the protein is sometimes called “AP” for amyloid P component (Pepys et al. 1994), although it has been shown that at least the protein core of AP is identical to SAP. The normal function of SAP is not clearly established, but one potential function of the glycoprotein is to bind plasma chromatin (Pepys 2001) released from necrotic and apoptotic cells (Paul and Carroll 1999). SAP knockout studies in a mouse model suggest that the glycoprotein offers protection against lupus—an autoimmune disease whose manifestations include the formation of antibodies against nuclear components such as DNA (Paul and Carroll 1999). SAP has additional procoagulant activity (Zahedi 1997), binds to components of the immune system (Gewurz et al. 1995), has chaperone activity in vitro (Coker et al. 2000), and is a component of a number of basement membranes (Zahedi 1997).

The three-dimensional structure of SAP belongs to the pentraxin family, which also includes C-reactive protein. The SAP monomers adopt an LNS-like β -jellyroll fold (Fig. 2). Native SAP glycoprotein in serum has a quaternary structure arranged in a homo-pentameric ring (Emsley et al. 1994). By contrast, isolated SAP in the presence of Ca²⁺ undergoes an aggregation and precipitation reaction. Aggregation of SAP is believed to be promoted by an (AB)_n face-to-face stacking of pentamers, where the A face corresponds to the side of the molecule that includes the longest α -helix (α -helix face), and the B-face (binding face) to the side of the monomer that includes the two Ca²⁺ ions (Fig. 2). The aggregation reaction is blocked by ligands that bind to SAP through its two Ca²⁺ ions, and by high salt. In the presence of small molecule ligands that bind to the double calcium site and inhibit aggregation, SAP gives decameric crystal structures resulting from pentameric rings

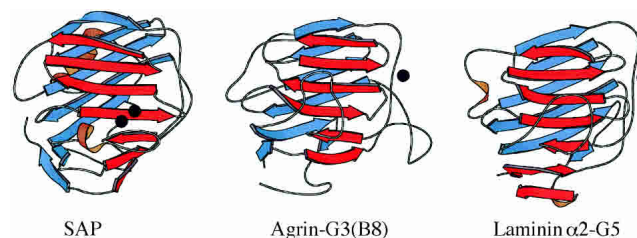


Figure 2. Examples of LNS/pentaxin motifs from SAP (Emsley et al. 1994), agrin (Stetefeld et al. 2004), and laminin (Hohenester and Engel 2002). The fold is a β -sandwich comprised of a convex (blue) and a concave (red) β -sheet. The LNS/pentaxin fold is distantly related to those of the C-type lectins. LNS-fold proteins have a broad range of binding partners ranging from proteins to carbohydrates to steroids (Rudenko et al. 2001). Despite similar folding topologies, metal and substrate binding sites are poorly conserved (Rudenko et al. 2001). For example, the double Ca²⁺ binding site in the SAP monomer structure occurs in an entirely different location than the Ca²⁺ binding sites in agrin and laminin. Note that the LNS motif of SAP is part of a homopentameric ring structure.

with B-to-B (dAMP) or A-to-B (MO β DG) arrangements (Thompson et al. 2002). Although a high-resolution structure of the apo protein is not yet available, SAP in the absence of Ca²⁺ is believed to have a decameric quaternary structure with an A-to-A arrangement of pentamers (Thompson et al. 2002).

SAP exists as a glycosylated protein in vivo. Its biantennary glycostructure is unusual in that its sequence is highly conserved (Pepys et al. 1994). SAP shows Ca²⁺-dependent binding to glycosaminoglycans such as heparan sulfate and dermatan sulfate (Hamazaki 1987), and to laminin (Zahedi 1997). Binding of SAP to amyloids also requires Ca²⁺ (Hamazaki 1987, 1995; Pepys 2001).

Agrin

The best understood function of the heparan sulfate proteoglycan agrin is its orchestration of acetylcholine receptor clustering on postsynaptic membranes (Sanes and Lichtman 2001; Bezakova and Ruegg 2003). The formation of these structures is a hallmark of neuromuscular junction development. Agrin is a mosaic protein comprised of ~22 domains, including nine protease inhibitor domains (Verbeek et al. 1999; Stetefeld et al. 2004). Splicing of agrin mRNA results in variants that include a globular N-terminal domain, which binds to laminin and anchors agrin to basement membranes. Alternatively the N-terminal domain can be swapped for a transmembrane segment that results in a lipid-membrane bound form of agrin (Bezakova and Ruegg 2003). Splice sites in agrin's globular LNS2 domain modulate binding to α -dystroglycan, an interaction thought to serve as a structural bridge between the basal lamina and muscle (Sanes and Lichtman 2001; Bezakova and Ruegg 2003). Additional mRNA splice sites generate neuron-specific isoforms of agrin, with inserts of eight to 18 residues in the last globular domain, LNS3. These insert containing forms are necessary for the acetylcholine receptor clustering activity of agrin (Bezakova and Ruegg 2003; Stetefeld et al. 2004). Besides agrin's functions in neuromuscular junction development, the proteoglycan is involved in synapse remodeling (Sanes and Lichtman 2001), in the formation of central nervous system synapses, and in the organization of "immunological synapses" (Khan et al. 2001; Bezakova and Ruegg 2003). Agrin is normally found in several areas of the brain, including neurons and in the basement membranes of brain microvasculature. Recently, an insoluble form of agrin was found to be highly concentrated in both diffuse and neuritic plaques of brains from patients with Alzheimer's disease (AD) (Donahue et al. 1999; Verbeek et al. 1999; van Horsen et al. 2001). Moreover, agrin was also associated with the neurofibrillary tangles characteristic of the disease (Donahue et al. 1999). The brains of AD patients showed basement membrane abnormalities, which may be related to the sequestration of agrin by amyloid (Donahue et al. 1999). Agrin in a B0 isoform is expressed in a variety of

tissues other than nerve cells (Bezakova and Ruegg 2003), and it remains to be seen if agrin-B0 associates with other types of amyloid fibrils besides those involved in AD.

In vitro studies (Cotman et al. 2000) indicate that agrin binds to fibrillar, but not to soluble A β . Binding of agrin accelerates A β -fibril formation, stabilizes the fibrils against proteolytic degradation (Cotman et al. 2000), and may indirectly be involved in A β toxicity through the abrogation of agrin's normal functions in synapse remodeling and microvasculature function (Donahue et al. 1999). Pretreatment of agrin with nitrous acid (conditions, and acid concentration were not given) almost completely diminished binding to fibrillar A β (Cotman et al. 2000). Retention of tenascin and merosin binding, suggested that only the GAG chains and not the protein core are affected by treatment of agrin with nitrous acid; and consequently, that agrin binds to A β fibrils through its GAG chains (Cotman et al. 2000). It is important to note, however, that agrin has ~22 protein domains that could also potentially be affected by the nitrous acid treatment. These domains include globular LNS domains (Fig. 2) that do not interact with tenascin or merosin, but that bind to GAGs in a Ca²⁺-dependent manner.

Perlecan

Perlecan is a basement membrane HSPG with roles in the adhesion, development, differentiation, and proliferation of cells (Hartmann and Maurer 2001; Jiang and Couchman 2003). In addition, perlecan interacts with a number of growth factors and participates in forming a semipermeable barrier in kidney glomerular basement membranes. Perlecan coimmunolocalizes with all types of amyloids (Snow and Wight 1989). Perlecan accelerates fibril formation (Castillo et al. 1998, 1999), stabilizes fibrils once formed (Castillo et al. 1997), and protects fibrils from proteolytic degradation (Gupta-Bansal et al. 1995).

Laminin

The large multidomain glycoprotein laminin is an integral component of basement membranes. In the presence of Ca²⁺, laminins polymerize into gel-like structures. These structures serve as a scaffold and recruit other components needed for basement membrane assembly during development. Studies using a mouse model suggest that laminin is the only factor absolutely required to form basement membranes during early stages of embryo development (Sasaki et al. 2004; Yurchenco et al. 2004). Additional functions of laminins include maintenance of basement membrane structure, and acting as anchors for proteins that associate with the basement membrane. Laminins are involved in a range of specific biological processes such as chemotaxis, mitosis, induction of tumor metastasis, and development of the peripheral nervous system (Engel 1992). The molecules are heterotrimers composed of α , β , and γ chains connected through a three-stranded heterotrimeric coiled coil. There

are five α , three β , and three γ chains that can form at least 15 different types of laminins (Sasaki et al. 2004). Electron microscopy of laminin (EHS) reveals a cross-like shape, with the longest α chain forming the central stalk (Engel 1992). At the end of this stalk, is a string of five globular LNS-type domains (Fig. 2; Engel 1992).

In contrast to the molecules discussed above, laminin (EHS) has been shown to inhibit amyloid formation (Castillo et al. 2000). Laminin-1 can also dissociate fibrils into protofibrils and further into amorphous aggregates, and has been reported to protect cultured neurons from A β -amyloid neurotoxicity (Morgan et al. 2002). The inhibitory effects appear to be specific to A β -amyloid, and were not observed with IAPP fibrils (Castillo et al. 2000). Digestion of laminin (EHS) with the protease elastase was used to map the binding site for A β fibrils to the last three globular domains of the α -chain (Castillo et al. 2000). Short peptides derived from laminin- α chain sequences upstream of the LNS domain also inhibit fibril formation, and may constitute a second binding domain for A β fibrils (Castillo et al. 2000; Morgan et al. 2002).

Apolipoproteins E and J

Apolipoprotein E (apoE) is a 34 kDa protein involved in lipid transport and metabolism. Together with plasma lipids, apoE assembles into lipoprotein complexes that are recognized by low-density lipoprotein (LDL) receptors (Strittmatter et al. 1993; Bales et al. 2002). Interestingly, the first step in the uptake of low-density lipoproteins by hepatocytes involves binding of ApoE to HSPGs (Fukuchi et al. 1998). In the central nervous system apoE is involved in the redistribution of lipids and cholesterol during membrane repair and synapse remodeling, and in the transport of lipids through the cerebrospinal fluid (Bales et al. 2002). Isoforms of apoE are genetic markers for late-onset Alzheimer's disease with alleles ϵ 4 and ϵ 2 increasing and decreasing the risk for the disease, respectively (Bales et al. 2002). The isoforms differ in only two amino acids (ϵ 2–Cys112, Cys 158; ϵ 4–Arg112, Arg 158), which affect the binding preferences for high-density lipoproteins (ϵ 2) versus low-density lipoproteins (ϵ 4). Carriers of the ϵ 4 allele have an additional predisposition for hypercholesterolemia (Bales et al. 2002).

ApoE (ϵ 3 or ϵ 4 isoform) tightly binds to soluble A β peptide forming complexes that resist dissociation after boiling for 5 min in 2% SDS (Strittmatter et al. 1993). ApoE also binds to A β in its fibril form; hence, antibodies to apoE colocalize with nearly all neuritic plaques in Alzheimer's disease brains (Bales et al. 2002). The importance of apoE in Alzheimer pathology is highlighted by the observation that transgenic mice carrying the gene for human APP, but lacking the gene for ApoE form only diffuse plaques but not the neuritic plaques and cerebral blood vessels amyloid deposits characteristic of the disease (Bales et al. 2002; Holtzman 2003).

In addition to its role in A β fibrillogenesis, apoE has a role in A β peptide clearance from the brain (Bales et al. 2002; Holtzman 2003). The second major brain apolipoprotein, apoJ (also called clusterin), binds soluble A β with a 2 nM K_d , and increases amyloid toxicity. Owing to its chaperone-like properties, apoJ solubilizes a large variety of proteins with exposed hydrophobic patches. The toxicity of ApoJ may arise from its ability to shift the equilibrium between fibrillar and soluble forms of the A β peptide toward the soluble forms, thereby unmasking toxic epitopes (DeMattos et al. 2002b).

Shared themes among the major proteins that interact with amyloid

All of the proteins described above are extracellular. Functionally, they can be loosely classified as proteins involved in the formation of supramolecular assemblies. ApoE forms lipoprotein complexes involved in lipid transport; the other four proteins participate in various aspects of basement membrane structure. Laminin is the main component of basement membranes. Together with laminin, agrin and perlecan share roles in basement membrane structure. As previously described, SAP in addition to circulating in the blood (Hind et al. 1984) is found associated with a number of basement membranes (Zahedi 1997). The link between amyloid deposits and the extracellular matrix (Kisilevsky 2000; Ancsin 2003; Bush 2003) is intriguing, given the importance of the latter to the function, association, and development of cells.

SAP, agrin, laminin, and perlecan share LNS/pentraxin β -jellyroll folding motifs as parts of their three-dimensional structures (Fig. 2). The LNS motif of SAP forms a pentameric ring structure. In agrin, laminin, and perlecan, LNS domains occur at the C-terminal ends of the multidomain proteins. LNS motifs occur in a number of functional contexts. The calcium- and substrate-binding sites of LNS domains are poorly conserved, making it difficult to draw functional inferences based on the presence of this folding motif (Rudenko et al. 2001). Nevertheless, SAP, agrin, laminin, and perlecan share at least one common functional aspect—binding to the GAGs heparin/heparan sulfate. Heparin binds to LNS domains 4 of laminin α 1, domains 3 and 5 of laminin α 2 (Talts et al. 1999), LNS4 of laminin α 4 (Yamashita et al. 2004), LNS2 of agrin (Burgess et al. 2002), and the three LNS domains within perlecan domain V (Brown et al. 1997). Affinity for heparin indicates a potential to bind to matrix bound heparan sulfate proteoglycans (Talts et al. 1999). Heparan sulfate binding has not been studied as extensively because it is harder to obtain (Kisilevsky 2000), but has been localized to the LNS domain pairs LG4–5 of laminin α 1 (Sasaki et al. 1998), and to the agrin LNS2 domain (Burgess et al. 2002). The N-terminal portions of laminin α 3B and α 5 chains, which do not contain LNS domains, have additional binding sites for

heparan sulfate (Garbe et al. 2002). SAP has been shown to bind to heparin, heparan sulfate, and dermatan sulfate (Hamazaki 1987). ApoE, whose structure unlike the other four proteins above does not include LNS motifs, binds to heparan sulfate proteoglycans as part of its function in lipid transport (Fukuchi et al. 1998; Libeu et al. 2001). These molecules, through additional interactions with components of basement membranes (Yurchenco et al. 2004), could anchor fibrils to the extracellular matrix, and provide a nidus for further amyloid growth. SAP, which associates with basement membranes and has shown to bind laminin with high affinity (Zahedi 1997) could conceivably play a similar role. Agrin and perlecan, which are heparan sulfate proteoglycans as well as having the capacity to bind heparan sulfate, could potentially further aggravate amyloid growth by forming intermolecular cross-links.

Other proteins associated with amyloids

In addition to the cofactors described in detail above, other molecules have been reported to interact with amyloid, specifically with amyloid formed by the Alzheimer's A β peptide. Predominant among these are basement membrane molecules including collagen IV and entactin (Kiuchi et al. 2002), the proteoglycans decorin, biglycan, versican (Snow et al. 1995), the heparan sulfate proteoglycans glypican-1, and syndecans 1 through 3 (van Horssen et al. 2002). It is interesting to note that Alzheimer's APP itself is classified as a "part time" proteoglycan (Bosman and Stamenkovic 2003) since it is sometimes found in forms with GAG chains attached. APP also has binding sites for HSPGs (Rossjohn et al. 1999; Wang and Ha 2004). Additional proteins reported to interact with A β amyloid that do not share readily apparent relationships, include the serpin antichymotrypsin (Lukacs and Christianson 1996), C1q A complement collagen-like domain (Wang et al. 2000), α 2 macroglobulin (Hughes et al. 1998), p75 neutrophin receptor (Yaar et al. 1997), and α 7 nicotinic acetylcholine receptor (Wang et al. 2000).

Cofactors in therapeutic approaches

Cofactors can affect the assembly, stability, and toxicity of amyloid fibrils. Each of these facets of amyloid etiology is a potential therapeutic target, and knowledge about the roles of cofactors in amyloid diseases has been exploited to develop new strategies for treatment. Efforts include the development of improved diagnostics and therapies for disease.

Diagnostics

Until recently, diagnosis of amyloid pathology depended on the analysis of samples from tissue biopsies or autopsies (Pepys 2001; Bacskai et al. 2003). The development of non-invasive diagnostics would allow better monitoring of dis-

ease progression and evaluation of treatments. Current diagnostics include small molecule probes, derived from amyloid-binding dyes such as Congo Red and Thioflavin (Lee 2002; Bacskai et al. 2003). Cofactors involved in the progression of the disease by comparison, offer the advantage of higher selectivity. The group of Pepys, has pioneered scintigraphy of amyloid using radioactively labeled ^{123}I -SAP tracers (Pepys 2001; Pepys et al. 2002). This approach takes advantage of the fact that SAP is associated with amyloid deposits. Following intravenous injection, ^{123}I -SAP is catabolized rapidly and cleared in the urine if no amyloid is present (Pepys 2001). When amyloid is present, ^{123}I -SAP from the blood exchanges through a dynamic equilibrium with the preexisting amyloid-bound SAP. The radioactive tracer provides quantitative information on the location and amount of amyloid (Pepys 2001). In turn, ^{123}I -SAP scintigraphy can be used to monitor dynamic turnover of amyloid, and can assess the efficacy of treatments aimed at regressing amyloid deposits (Pepys et al. 2002). For some applications, it would clearly be desirable to have multiple diagnostic probes. Radioactive or spin-labeled analogs of agrin that cross the blood-brain barrier may have applications for detecting amyloid deposits in Alzheimer's disease patients (Gurwitz 2000).

Interference with amyloid facilitators

Highly sulfated GAGs are ubiquitous in amyloid deposits, and participate in the assembly of fibrils. Compounds that compete with the binding of GAGs or proteoglycans to the amyloid precursor proteins have shown effectiveness in blocking fibril assembly (Kiselevsky et al. 2003). These compounds include chain terminators that once incorporated into growing GAG chains block further elongation, and carbohydrate precursors that cannot be properly sulfated thus abrogating the requirement for highly negatively charged polyanions for efficient amyloid binding (Kiselevsky et al. 2003). In another approach, sulfated polyanion mimetics of heparan sulfate such as poly(vinylsulfonate) or derivatives of dextran sulfate were used to interfere with SAA fibril assembly (Inoue et al. 1996) and to block prion protein propagation (Adjou et al. 2003). Additional strategies for interfering with amyloid assembly include mimetics of epitopes on proteins such as laminin that promote fibril disassembly (Castillo et al. 2000), and inhibition of the expression of molecules such as apoE which promote fibril formation (Bales et al. 2002).

Targeting the amyloid enforcer SAP

A different strategy targets molecules that stabilize amyloids to degradation in vivo. Early studies showed that a trace component of agarose, methyl 4,6-O-(1-carboxyethylidene)- β -D-galactopyranoside (MO β DG) inhibited the Ca^{2+} -dependent binding reactions of SAP, including the binding of SAP to amyloid (Hind et al. 1984). Presumably

this compound mimics the Ca^{2+} -dependent affinity of SAP for glycosaminoglycans such as heparan sulfate and dermatan sulfate (Hamazaki 1987). At millimolar concentrations, MO β DG displaces SAP from amyloid and returns it to the blood where it can be transported to the liver for catabolic clearance (Hind et al. 1984; Pepys 2001). Amyloids regress as the fibril-stabilizing and protease-resistance-conferring SAP molecules are removed (Pepys 2001). A high-throughput screen of a compound library was used to identify CPHPC, a new competitive inhibitor of SAP that interferes with amyloid binding (Pepys et al. 2002). Compared to the 50- μM affinity constant for MO β DG, the apparent affinity constant for the CPHPC compound is 10 nM. This value is comparable to the 10-nM dissociation constant estimated for binding of SAP to isolated amyloid fibrils. X-ray structures of the SAP complexes with MO β DG (Thompson et al. 2002) and CPHPC (Pepys et al. 2002) show that the compounds bind near the double- Ca^{2+} ligation site of the protein, and thus probably work by interfering with the Ca^{2+} -dependent binding of SAP to amyloid (Thompson et al. 2002). Both compounds stabilize decameric forms of SAP, which are rapidly cleared from circulation by the liver (Pepys et al. 2002).

Shifting the partition of A β across the blood–brain barrier

Antibodies raised against A β peptides offer a potential therapeutic approach to Alzheimer's disease by reducing the load of A β in the central nervous system (Schenk et al. 1999). A β synthesized in the brain can be catabolized locally, or transported into the plasma and cleared peripherally (Bales et al. 2002; DeMattos et al. 2002a). Using a mouse model it has been shown that a peripherally administered antibody to A β can act as a "peripheral sink" that by binding A β in the blood markedly increases the efflux of A β from the brain to the plasma (DeMattos et al. 2002a). The "peripheral sink" approach could find more general utility with other molecules that bind to plasma A β (Matsuoka et al. 2003) and lack some of the adverse effects associated with A β -directed antibodies.

Alleviation of metal-induced toxicity

The mechanism by which amyloid deposits exert their pathologic effects remains unresolved, but evidence suggests a role for metals in at least some aspects of amyloid toxicity (Bush and Tanzi 2002; Bush 2003). Nonspecific high-affinity chelators that reduce the overall concentration of metals in the body are unlikely to be successful in treating amyloidoses, because toxicity occurs in the specific context of amyloid-bound metals (Bush 2002, 2003). Moreover, toxicity is localized to the tissues neighboring amyloid deposits, such as the brain in AD. Success has been reported in a mouse model for AD with clioquinil, a retired antibiotic that serves as an orally deliverable Cu–Zn chelator. The

hydrophobic character of Clioquinil enables it to cross the blood–brain barrier, in contrast to more common hydrophilic metal chelators. The compound reduces A β deposits by ~50% in mice, and is thought to work by simultaneously facilitating the dissolution of Zn $^{2+}$ -bound amyloid and inhibiting copper and iron-mediated H $_2$ O $_2$ production. Inhibition of H $_2$ O $_2$ further depletes A β by an indirect clearance mechanism (Bush 2003). Once resolubilized, A β peptides can be removed in the blood or taken up by cells for degradation (Bush 2003). Because clioquinil has a relatively weak nanomolar affinity for zinc and copper, it does not induce systemic metal depletion as would be the case with stronger chelators (Bush 2002).

Open questions regarding the biophysics of amyloids

This review has summarized the roles of accessory molecules in the biology of amyloid diseases. Much remains to be understood about the biophysics of amyloid–cofactor interactions: How large of an effect do cofactors have on the structures and stabilities of amyloids? To what extent do the core protein and carbohydrate components of glycoproteins and proteoglycans participate in amyloid recognition? What are the structural determinants of cofactor binding to amyloids? Addressing these questions should lead to a better appreciation of the role of cofactors in the development of amyloid disease and will undoubtedly contribute to a better understanding of the mechanisms of amyloid toxicity.

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