REVIEW

Amyloid—a state in many guises: Survival of the fittest fibril fold

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Abstract

Under appropriate conditions, essentially all proteins are able to aggregate to form long, well-ordered and β -sheet-rich arrays known as amyloid-like fibrils. These fibrils consist of varying numbers of intertwined protofibrils and can for any given protein exhibit a wealth of different forms at the ultrastructural level. Traditionally, this structural variability or polymorphism has been attributed to differences in the assembly of a common protofibril structure. However, recent work on glucagon, insulin, and the A β peptide suggests that this polymorphism can occur at the level of secondary structure. Simple variations in either solvent conditions such as temperature, protein concentration, and ionic strength or external mechanical influences such as agitation can lead to formation of fibrils with markedly different characteristics. In some cases, these characteristics can be passed on to new fibrils in a strain-specific manner, similar to what is known for prions. The preferred structure of fibrils formed can be explained in terms of selective pressure and survival of the fittest; the most populated types of fibrils we observe at the end of an experiment are those that had the fastest overall growth rate under the given conditions. Fibrillar polymorphism is probably a consequence of the lack of structural restraints on a nonfunctional conformational state.

Keywords: amyloid; fibril fold

Ubiquitous amyloid and strain polymorphism

With few exceptions (Luo et al. 2004), most proteins fold into an active, singular folded conformation based on their amino acid sequences (Anfinsen 1973). These sequences have been optimized through millions of years of evolution with gatekeeper residues to avoid various folding pitfalls (Otzen and Oliveberg 1999; Otzen et al. 2000). In fact, most protein can accept large sequence perturbations without changing the overall protein fold (Fersht 1999; Gazit 2002). It is, however, becoming

increasingly apparent that proteins also have an intrinsic ability to misfold and aggregate into highly ordered and elongated nanoscale cross- β structures known as amyloid-like fibrils (Dobson 1999; Sipe and Cohen 2000). This tendency is highly modulated by the amino acid composition (i.e., sequences rich in hydrophobic aromatics and β -branched generally have a high amyloid forming tendency) (Pawar et al. 2005), and several prediction algorithms have proven successful in predicting sequences prone to fibril formation (Fernandez-Escamilla et al. 2004; Thompson et al. 2006; Hamodrakas et al. 2007). Because of the stable hydrogen-bonded β -rich structure of amyloid (Makin et al. 2005), it has been suggested that the single-fold dogma seen for native proteins could also apply to these highly ordered aggregates. However, while amyloid fibrils, viewed in the electron microscope, generally share an elongated unbranched fibrillar appearance, they often

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come as mixtures of different morphologies, as illustrated by glucagon fibrils in Figure 1. Fibrils of almost identical morphologies can be formed by unrelated proteins, such as insulin (Jimenez et al. 2002), calcitonin (Bauer et al. 1995), amylin (Goldsbury et al. 1997), and A β_{1-40} (Goldsbury et al. 2000).

Traditionally, this polymorphism has often been interpreted as differences in the packing of a common building block, the protofilament, in the mature fibril (Bauer et al. 1995; Goldsbury et al. 1997, 2000; Serpell et al. 2000; Jimenez et al. 2002). One theory is that the formation of fibrils proceeds in a hierarchical fashion, starting with formation of critical oligomers that aggregate to form protofibrils, which then gradually come together to form mature fibrils (Modler et al. 2004). However, different growth rates observed for A β protofibrils and fibrils, which both appear to consist of two protofilaments (identical mass per length), are inconsistent with this model, and suggest that the different structures are formed via individual pathways (Goldsbury et al. 2005). Moreover, recent studies have shown that several proteins including A β , α -synuclein, glucagon, and insulin are capable of forming mature fibrils with highly variable characteristics, such as differences in staining with amyloid specific dyes, thermostability, and secondary structure as judged by CD and FTIR (Nielsen et al. 2001a; Pedersen et al. 2006a,c), as well as differences in molecular packing as judged by ssNMR (Heise et al. 2005; Petkova et al. 2005). Moreover, fibril characteristics can be passed on by seeding in a strain-like behavior (Dzwolak et al. 2004; Petkova et al. 2005; Dzwolak 2006; Pedersen et al. 2006a,c) as seen for the prions (Bessen et al. 1995). Recent results indicate that strain behavior is a consequence of differences in the molecular packing of prion proteins (Tanaka et al. 2004), and that specific

environmental conditions appear to provide growth advantage to specific strains in a survival-of-the-fittest manner (Tanaka et al. 2006). Because strain behavior is observed for proteins as disparate as insulin (Dzwolak et al. 2004), A β (Petkova et al. 2005), glucagon (Pedersen et al. 2006a), and prions (King and Diaz-Avalos 2004), we suggest that differences in molecular packing also underlie the polymorphisms observed for non-prion amyloid. Here we review how survival-of-the-fittest mechanisms can cause the preferred amyloid molecular packing to correlate with the conditions present under fibril formation (Petkova et al. 2005; Pedersen et al. 2006a).

Survival of the fittest?

When grown under a specific set of conditions, the fibrils formed exhibit a high degree of order (Jaroniec et al. 2002; Diaz-Avalos et al. 2003; Knowles et al. 2006). Although the presence of a high degree of order alongside structural polymorphism may seem like a contradiction of terms, the actual phenomenon of structural polymorphism is, on reflection, not entirely unexpected. Since the molecular structure of the amyloid state in all but a few cases (Kelly and Balch 2003) does not serve a purpose for living organisms, peptides that form amyloid have not been optimized through evolution to fold into one unique structure. This means that the single-fold dogma seen for most native proteins does not necessarily apply to amyloid-like aggregates. The structural basis for polymorphism may in many cases involve different packing interactions between β -strands. A very recent landmark paper by Eisenberg and coworkers reports microcrystalline fibril structures of 10 different proteins (Sawaya et al. 2007). Three of these form two different fibrillar arrangements, which arise either by changes in the orientation of the two fibril faces (e.g., “up–up face-to-face” versus “up–down face-to-back”) or subtle changes in the side-chain orientations, leading to different packing of the steric zipper within the crystal. Multiple possibilities for zipper contacts will also lead to different possible ways of packing the β -sheets, ultimately forming polymorphic fibrils.

But why are amyloid-like structures highly ordered? Consider a familiar biological system consisting of a small inoculate of different bacteria growing in a flask containing media. If this media is rich, we expect that the resulting “end” culture will probably consist of a mixture of different bacteria, with population numbers of individual bacteria strains depending on how fast they grow and the number present in the initial inoculation (founder effects). However, if we change the media to more challenging conditions (e.g., remove certain components, or add antibiotics) we exert a selective pressure on the system, resulting in impaired growth or even death for

Figure 1. Examples of some of the different morphologies observed for glucagon fibrils formed at pH 2–3. The scale bar is 50 nm. For the parallel bundled fibrils observed in a single batch, the apparent protofilament widths range from 5.2 to 6.1 nm.

some bacteria, giving tremendous growth advantage to more resilient species. Thus the population distribution of the end culture may be changed significantly by changes in the media. In fact, it may be completely dominated by the fastest growing type of bacteria and thus considered a highly “ordered” solution.

Based on studies on the fibril formation of the 29-residue peptide hormone glucagon, we suggest that the appearance of several different types/strains of amyloid fibrils from one polypeptide sequence may be explained by the same basic principles of survival of the fittest that govern the growth of bacteria in a flask of media. Here the fibrils would grow by “consuming” monomeric proteins/peptides present as “media.” If the exponential growth rate of individual types of fibrils is dependent on assay conditions, then even small variations in conditions could change the resulting fibril population distribution. As is discussed in the following sections, these variations can arise from both changes in solvent conditions, protein concentration, agitation, and protein sequence. Although these considerations can also affect proteins that have a well-defined structure under physiological conditions, we note that our considerations are particularly pertinent to proteins whose structures are dynamic and flexible from the onset, that is, peptides and natively unfolded proteins.

Alteration of the protein sequence leading to different fibril types

It has been known for a long time that fibril formation is very specific and that like proteins can pair up even in crowded mixed environments (London et al. 1974). Conversely, even small changes in the protein sequence can dramatically reduce their ability to elongate wild-type fibril seeds (O’Nuallain et al. 2004), although in other contexts homologous proteins appear able to cross-seed, provided they have a threshold similarity of at least 30% (Krebs et al. 2004). The flip side of this property is that individual mutations can have large consequences on the structure of the aggregates, such that a single mutation can sway the balance from ordered fibrils to aggregates (Helms and Wetzel 1996). Similar changes can be observed for glucagon, where alanine-scanning mutations of some residues result in dramatic changes in the fluorescence spectrum of the resulting fibrils (Pedersen et al. 2006b). A direct physiological consequence is seen in the case of Transforming Growth Factor β -induced Protein (TGF β Ip) involved in corneal dystrophies, where one mutation (e.g., A546T) can lead to amyloid-type deposits, whereas mutations such as R555W and R555Q lead to amorphous deposits, with different clinical profiles (Munier et al. 2002). This observation is not simply due to different interactions with the cellular apparatus, since the effects are reproduced in vitro (R.V. Basaiawmoit, K.

Runager, J. Enghild, and D.E. Otzen, unpubl.). This suggests that the mutation changes the structure of the fibrils formed, and hence one should exercise extra caution when trying to draw conclusions about the structure of amyloid fibrils from mutagenesis studies.

The preferred fibrillation mechanism depends on protein concentration

It is widely established that many proteins form amyloid fibrils via nucleation-dependent growth mechanisms (Chiti and Dobson 2006). The time course for this type of growth typically includes a lag phase, followed by an exponential growth phase, which terminates in an equilibrium phase that sets in upon depletion of monomeric protein/peptide. Fibril formation is initiated by nucleation event(s), after which individual fibrils grow by linear elongation via addition of molecules to the end(s). Because the nucleus often consists of several molecules that represent an unfavorable high-free-energy species (Ferrone 1999), nucleation rates are often highly dependent on protein concentration. High protein concentrations can therefore often accelerate nucleation, but may also result in formation of both on- and off-pathway oligomeric intermediates (Kayed et al. 2003; Ahmad et al. 2005; Dong et al. 2006; Andersen et al. 2007). In contrast, fibril nucleation can be a very rare event at low concentrations; here, fibrils capable of increasing their number of fibril ends through secondary pathways have tremendous selective advantage because they can grow exponentially (Ferrone 1999). Secondary pathways include fragmentation or internal scissions (Hall and Edskes 2004), branching, or heterogeneous nucleation (Ferrone et al. 1985) (i.e., catalysis of nucleation by existing fibrils). For specific types of glucagon fibrils, simple seeding experiments readily demonstrate that the exponential growth phase extends into the lag phase (which should as a consequence be referred to as a log phase), back to the very start of the experiment (Pedersen et al. 2006a) (see original data in Fig. 2). This means that the majority of the fibrils present at the end are descendants of very few early nucleation events shortly after the protein was dissolved. As a further consequence of this, any oligomeric intermediate species formed in the course of the lag phase must be regarded as either nonessential or off-pathway with regard to formation of this type of fibril, but may decrease the concentration of monomers available and thus reduce growth rate. Because the selective advantage of efficient secondary pathways may require specific structural properties, the fibrils formed at higher protein concentrations (with more rapid nucleation) need not have the same structural properties as those formed at low concentrations. In fact, it has been demonstrated that the structure of glucagon fibrils depends on protein

Figure 2. Time course for seeded glucagon fibril formation. One gram per liter of glucagon in 50 mM glycine/HCl (pH 2.5) is seeded with a threefold dilution series of preformed fibrils. The mass fraction of preformed fibrils is indicated on the logarithmic Y-axis of the graph. The exponential growth phase extrapolates to (just above) the seed fraction added. Below seed fractions of 1.5×10^{-5} , spontaneous nucleation accounts for more fibril formation than elongation of seeds. However, the shape of the observed fibrillation curve remains unchanged. At higher seed fractions the slope of the curve increases, which can be attributed to a shorter average length of the seeding fibrils relative to the average length of fibrils during the log phase.

concentration: Fibrils formed at high concentrations stain intensively with ThT (fibril type A) and appear as thin flexible fibrils in EM (Pedersen et al. 2006a; Andersen et al. 2007). In contrast, fibrils formed at low concentrations (fibril type B) stain very weakly with ThT, and appear as thicker, bundled fibrils in EM (Pedersen et al. 2006a). Furthermore, type B fibrils have an unusual β -turn-like fingerprint in the CD spectrum as well as a distinct peak in the infrared spectrum and are much more resistant to thermal denaturation than type A (see Fig. 3C; Pedersen et al. 2006a). Profound differences in the heat capacity of the different fibril types have also been demonstrated by Isothermal Titration Calorimetry (M.D.J. Jeppesen, P. Westh, and D.E. Otzen, unpubl.), while X-ray fiber diffraction highlights subtle changes in the β -strand distances (C.A. Andersen, L. Serpell, and D.E. Otzen, unpubl.). Such observations could be taken to indicate the existence of different β -sheet conformations for the same sequence, just as the Ramachandran plot shows a range of allowed β -sheet conformations.

Heterogeneous nucleation and stabilization of fibrils by ions

Several proteins, including S6 (Pedersen et al. 2004), mouse V_L domain (Martsev et al. 2002), and lysozyme (Krebs et al. 2000), form amyloid much more rapidly by incubating them at low pH. Under these conditions, acidic side chains become protonated, and therefore proteins will generally only contain positive changes resulting in

Figure 3. (Legend on next page)

Coulombic repulsion of like charges. As a result, the structure of protein fibrils will depend on charge distribution (Wood et al. 1996; Smith et al. 2006). Addition of salts can increase rates of fibril growth both due to hydrophobic effects and Coulombic shielding of like charges (Munishkina et al. 2004; Raman et al. 2005). However, the addition of salts can also affect both secondary pathways and nucleation rates: When glucagon is incubated with more than ~ 0.1 M NaCl, type D fibrils start to outcompete type B fibrils (Pedersen et al. 2006a). Type D fibrils also exhibit low ThT fluorescence, but have a classical β -sheet signature with a negative peak at 218 nm (see Fig. 3A; Pedersen et al. 2006a). These inferred differences in secondary structure can also be observed as distinctive fingerprint peaks in the infrared spectrum (see Fig. 3B). The structural differences become evident in cross-seeding experiments, where the shape of the seeded fibrillation curve depends on the type of seed used (see Fig. 4). This suggests large differences in the growth mechanism of the two types (Pedersen et al. 2006a); that is, type B fibrils grow via fractionation, whereas type D probably grows by heterogeneous nucleation or branching (Ferrone 1999). As confirmed by CD spectroscopy, new fibrils formed inherit properties of the parent used for seeding (Pedersen et al. 2006a).

Not just the ionic strength, but also the type of ions present in the solution can have a dramatic impact on the preferred structure of fibrils: Addition of only 1 mM SO_4^{2-} can result in formation of “sulfate-type” glucagon fibrils, which is characterized by an α -helix-like fingerprint in the CD spectrum and a high ThT fluorescence (Pedersen et al. 2006c). Sulfate-type fibrils appear to require these negative anions to coordinate the five positive charges (i.e., N terminus, His1, Lys12, Arg17, and Arg18), since stability of the fibrils, as judged by the apparent thermal melting midpoint temperature (T_m^{app}), increases by a staggering 22°C for every 10-fold increase in sulfate concentration (Pedersen et al. 2006c). Sulfate groups are found naturally in glucosaminoglycans like heparin, which stimulates fibrillation of many different proteins (Snow et al. 1987). Sulfates also appear critical for the secondary pathways of sulfate-type fibril forma-

tion; although sulfate-type seeds can be extended at low salt conditions, they only appear to grow linearly (Pedersen et al. 2006c). This means that they are easily outgrown by other types of fibrils (e.g., types B and D) that grow exponentially at low sulfate concentrations. The different fibril types are summarized in Figure 5 and Table 1.

Agitation results in selective enrichment of brittle fibrils

Selection can also be exerted by external factors such as agitation. Agitation has been reported to accelerate the fibril growth for several proteins, including insulin (Nielsen et al. 2001b), glucagon (Pedersen et al. 2006a), S6 (Pedersen et al. 2004), and prions (Collins et al. 2004). It has been speculated that agitation could increase the nucleation rate by promoting unfolding in the hydrophobic air–water interface (Nielsen et al. 2001b). However, the fact that a simple end-over-end rotation can drastically increase the seeding capability of a tube containing fibrils formed without agitation (Collins et al. 2004), demonstrates that fibril fractionation can be promoted by agitation. A self-propagating, molecular-level polymorphism has recently been demonstrated for $\text{A}\beta_{1-40}$, depending on whether agitation is applied during fibril formation (Petkova et al. 2005): “Quiescent” fibrils appear as twisted in the electron microscope, whereas “agitated” fibrils invariably exist as untwisted parallel bundles of two or more filaments. Peak-shifts in the solid-state NMR spectra suggests that the packing of $\text{A}\beta$ peptides is different in the two fibril types (Petkova et al. 2005; Paravastu et al. 2006). Strikingly, exactly the same agitation-dependent morphologies can be observed for glucagon fibrils formed at low concentration (agitated [Pedersen et al. 2006a]; quiescent [Andersen et al. 2007]). The fact that these two morphologies are seen for unrelated proteins suggests that similar quiescent/agitated morphologies may also be found for the fibrils of several other proteins. The occurrence of different fibrils can again be interpreted via the survival-of-the-fittest dogma: If one type of fibril is so brittle that it breaks upon agitation, thereby doubling the number of exposed free ends, then this would accelerate secondary pathways and give this fibril type a tremendous selective growth advantage, compared to other, more flexible types of fibrils that do not break. Indeed, it has been demonstrated that the prion strains with the strongest phenotypes are those that have exhibited the highest level of brittleness (Tanaka et al. 2006).

One sequence, many structures?

Some researchers have suggested that the ability of $\text{A}\beta$ to form several different types of fibrils could be controlled

Figure 3. Overview of the spectroscopic properties of different glucagon fibrils. (A) CD spectra of sonicated fibrils compared to monomeric glucagon, showing the characteristic positive peak at 205 nm for type B (Pedersen et al. 2006a) and the α -helix-like spectrum of sulfate-type fibrils (Pedersen et al. 2006c). The unusual spectra may be caused by overrepresentation of aromatic residues in glucagon. (B) Comparison of FTIR spectra of dried samples of different glucagon fibrils, showing both the actual spectra and deconvoluted peaks. Data taken from Pedersen et al. (2006a,c). (C) Comparison of melting curves for sonicated fibrils of types A, B, C, and D. Data adapted from Pedersen et al. (2006a).

Figure 4. Seeding of 1 g/L glucagon in 50 mM glycine/HCl with fivefold dilution series of sonicated fibrils. (A) Seeding with type B fibrils: At high seed fractions, daughter fibrils inherit the low ThT staining of the parent. However, at lower seed fractions, there is a transient formation of the highly ThT-fluorescent type A fibril. (B) Seeding with type D fibrils: Curve shape is dramatically different from that obtained with type B seeds. Transient formation of type A fibrils is only observed at the lowest seeding fractions. (C) Analysis of kinetics indicates that type B and D fibrils have a growth rate of 0.33 and 0.48 h^{-1} , respectively, based on the time it takes for fibrils to grow to a fixed threshold. Data taken from Pedersen et al. (2006a).

by the unstructured N-terminal region (Kheterpal et al. 2001; Petkova et al. 2002). However, $A\beta_{10-40}$ exhibits the same type of fibrillation behavior as full-length $A\beta$ peptide, and even cross-seeds with $A\beta_{1-40}$ fibrils, demonstrating that the flexible N terminus of $A\beta$ is not involved in the fibrillar polymorphism (Paravastu et al. 2006). This means that structural differences lie in the β -aggregating core regions. The $A\beta_{1-40}$ and $A\beta_{10-35}$ fragments of the Alzheimer's peptides have been shown by solid-state NMR to form fibrils with parallel β -sheet packing (Antzutkin et al. 2000; Benzinger et al. 2000), whereas the fragments $A\beta_{16-22}$ and $A\beta_{34-42}$ form antiparallel fibrils (Lansbury Jr. et al. 1995; Balbach et al. 2000). Of the four mentioned peptides, the first two are predicted to be amphiphilic (i.e., hydrophobic at one end and hydrophilic at the other), whereas the last two are nonamphiphilic. A recent solid-state NMR study has shown that increasing the amphiphilicity of the $A\beta_{16-22}$ fragment chemically by acylation causes a change in fibril packing from antiparallel fibrils to parallel fibrils (Gordon et al. 2004). Since the relative amphiphilicity of a peptide may be perturbed by solvent conditions (e.g., the protonation state of charged residues will change with pH, and charges will be shielded by salts), changes in solvent conditions might also be able to cause large structural changes. In the light of the recent discoveries of polymorphic fibrils, it may be wise not to rule out the possibility for one orientation, just because the other has been demonstrated experimentally (Antzutkin et al. 2000).

Glucagon is slightly amphiphilic, but has two hydrophobic patches involved in fibril formation (Pedersen et al. 2006b), namely, one in the N terminus composed of residues Phe6, Tyr10, Tyr13, and Leu14, and a slightly more hydrophobic C terminus composed of Phe22, Val23, Trp25, Leu26, and Met 27. Thus, both parallel and antiparallel packings of glucagon fibrils could be imagined. While the in-register parallel conformation is generally expected to maximize hydrophobic interactions (Fig. 6, see below), it suffers large penalties from the short interstrand distances between like charged residues (Mattice 1989; Merkel et al. 1999). In a low dielectric environment, such as might exist in the hydrophobic core of fibrils, these electrostatic repulsions may destabilize the fibrils with as much as $\sim 100 \text{ kcal/mol}$ (Petkova et al. 2002). Consequently one would expect that the parallel types of fibrils would benefit significantly more from increased concentrations of charge-neutralizing ions than the antiparallel ones. Since type B fibrils are quite stable even at low salt conditions and the sulfate type (and possibly type D) appear to require negative ligands for stability, it is tempting to speculate that type B fibrils could be antiparallel, while sulfate-type fibrils could have parallel configuration of β -sheets. However, further studies on the structures of glucagon fibrils are required to test this hypothesis.

Figure 5. Summary on how selective environmental conditions affect the preferred secondary structure of glucagon fibrils. Based on kinetics data obtained from urea and proteolysis dissociation of fibrils (Pedersen et al. 2006a), we conclude that even the most stable glucagon fibril is in equilibrium with monomers. Therefore, transition from one type of fibril to another proceeds via recycling of monomers as suggested for the SH3 domain (Carulla et al. 2005).

Conclusions

When knowledge of the prions first started to emerge, researchers were intrigued by the appearance of several different strains in a protein-only world and the violation of the single-fold dogma (Bussard 2005). It is now becoming more and more clear that proteins do not require a prion-like sequence to have several associated states. Indeed, it is fascinating to see that even a simple biological molecule of only 29 residues appears to give new meaning to the famous words of Charles Darwin: “It is not the strongest of the species that survive, nor the most intelligent, but the ones most responsive to change.” Although not particularly desirable for any living organism, it appears that simple peptides can also be very responsive to change. Thus, if we attempt to prevent a protein from forming one particular type of fibrillar aggregate by blocking its nucleation or secondary pathways, the protein may simply start to form amyloid with an alternative packing through an alternative mechanism. The preferred amyloid-like structure of a given protein appears to depend on how environmental conditions affect both nucleation and fibril growth rates. However, even a slowly nucleating fibril may eventually turn out as the fittest fibril because of effective secondary pathways resulting in rapid exponential growth. We have so far demonstrated at least four different types of mature fibrils for our model protein, glucagon. By carefully correlating fibrillation kinetics with the resulting morphology and

secondary structure of fibrils during gradual adjustments in fibril growth conditions, we have been able to establish conditions that favor formation of each of these fibril types (Pedersen et al. 2006a,c). Based on the morphological similarities with fibrils of other proteins, we suggest that similar amyloid packings are available for proteins in general irrespective of primary structure. Thus, it appears that the formation of amyloid resembles plastic polymers more than native proteins (Kodali and Wetzel 2007).

In the light of the apparent general degeneracy of the nonnative β -aggregated structures that are collectively referred to as amyloid-like fibrils, we recommend that researchers do not use generalizing phrases like “⟨my protein⟩ forms fibrils of ⟨some structure⟩ via ⟨some mechanism⟩,” but instead, rephrase the expression to “under the given conditions, ⟨my protein⟩ predominantly forms fibrils of ⟨some structure⟩ via ⟨some mechanism⟩.”

Figure 6. Schematic representation of the distance between a charged residue at one particular position in a parallel and an antiparallel amyloid β -sheet.

Table 1. Summary of the different types of glucagon fibrils observed, and the conditions under which they form

Fibril type	Recipe conditions	Characteristics
Type A (Pedersen et al. 2006a; Andersen et al. 2007)	>1 g/L glucagon concentration, 50 mM glycine/HCl (pH 2.5)	Thin flexible fibrils, sometimes as twisted bundles; high ThT staining
Type B (Pedersen et al. 2006a)	<0.5 g/L glucagon, 50 mM glycine/HCl (pH 2.5), agitation	Untwisted parallel bundles of two or more filaments; low ThT staining; CD-spectrum fingerprint at 206 nm and characteristic IR peak at $\sim 1635\text{ cm}^{-1}$
Type B _{quiescent} (Andersen et al. 2007)	<0.5 g/L glucagon, 50 mM glycine/HCl (pH 2.5), no agitation	Twisted thick fibrils; low ThT staining
Type C (Pedersen et al. 2006a)	<0.5 g/L glucagon, 50 mM glycine/HCl (pH 2.5), agitation, elevated temperature (50°C)	Low ThT staining; large, untwisted parallel bundles of several filaments; similar molecular structure as type B
Type D (Pedersen et al. 2006a)	<0.5 g/L glucagon, 50 mM glycine, 100–250 mM NaCl (agitated)	High pitch twisting; low thioflavin T staining
Type S (Pedersen et al. 2006c) (Sulfate type)	$\sim 0.5\text{ g/L}$ glucagon, 1 mM SO_4^{2-} , 5–10 mM HCl (pH 2–2.5), agitation not required	Regular twisted fibrils; high ThT staining; irregular, α -helix-like CD spectrum

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