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A Role For Helical Intermediates in Amyloid Formation By Natively Unfolded Polypeptides?

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Abstract

Amyloid formation and aberrant protein aggregation have been implicated in more than 15 different human diseases and an even wider range of proteins form amyloid *in vitro*. From a structural perspective the proteins which form amyloid can be divided into two classes: those which adopt a compact globular fold and must presumably at least partially unfold to form amyloid and those which are unstructured in their monomeric state. Important examples of the latter include the A*β* peptide of Alzheimer's disease, atrial natriuretic factor, calcitonin, pro-calcitonin, islet amyloid polypeptide (IAPP, amylin), *α*-synuclein and the medin polypeptide. The kinetics of amyloid assembly are complex and typically involve a lag phase during which little or no fibril material is formed, followed by a rapid growth stage leading to the *β*-sheet-rich amyloid structure. Increasing evidence suggests that some natively unfolded polypeptides populate a helical intermediate during the lag phase. We propose a model in which early oligomerization is linked to helix formation and is promoted by helix–helix association. Recent work has highlighted the potential importance of polypeptide membrane interactions in amyloid formation and helical intermediates appear to play an important role here as well. Characterization of helical intermediates is experimentally challenging but new spectroscopic techniques are emerging which hold considerable promise and even have the potential to provide residue specific information.

1. Introduction

Amyloid formation plays an important role in a wide variety of human diseases including Parkinson's disease, Alzheimer's disease, the spongiform encephalopathy's and type 2 diabetes [1-3]. Furthermore, a wide range of proteins and peptides that do not form amyloid *in vivo* can be induced to do so in *vitro* and this has lead to the hypothesis that the ability to form amyloid is a generic property of polypeptide chains and a specific primary sequence is not required [4]. All amyloid fibrils share a number of common structural features despite the considerable diversity in primary sequence of the constituent proteins. Amyloid fibrils are typically long, unbranched and on the order of 5 to 20 nm in width. They are rich in β-sheet structure and the ordered regions adopt the classic “cross-β” structure in which individual strands in the β-sheets run perpendicular to the long axis of the fibril with the inter β-sheet hydrogen bonds thus oriented parallel to the fibril axis [5, 6]. The individual

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strands within the β-sheets can be parallel or anti-parallel although they are parallel in islet amyloid polypeptide (IAPP, also known as amylin) and in Aβ, the two polypeptides which form amyloid *in vivo* for which the most detailed structural information is available [7, 8]. The super molecular structure of fibrils can be complex and individual fibrils are built up from protofilaments which normally contain two or more β-sheets, with typically from 2 to 9 protofilaments per fibril

Early work focused on the characterization of fibril structure, and indeed this is still an extremely active and exciting area of research, as is the development of inhibitors of fibril formation. However, the hypothesis that intermediates in fibril assembly may be the most toxic species has emerged in the last few years and this has focused attention on the mechanism of amyloid formation and on the nature of intermediate species [9-13]. The characterization of intermediate species populated during amyloid formation has emerged as a major topic of biophysical research. In this paper we examine the biophysical literature on *in vitro* amyloid formation by natively unfolded polypeptides and propose that helical oligomeric intermediates play an important role in many systems.

Intense interest in intermediates has arisen both because of their mechanistic importance and because of the potential role of pre-amyloid intermediates in cellular toxicity. Structural characterization of any intermediate species that are formed along the pathway of amyloid formation is also clearly important for the rational design of inhibitors of amyloid formation. Unfortunately the details of amyloid formation are still not well understood despite considerable effort. The time course of amyloid formation is complex and normally involves a lengthy lag phase during which little fibril material is generated but during which a critical nucleus is formed [14-18]. The lag phase is followed by a rapid growth phase leading to the production of amyloid fibrils.

Proteins which form amyloid can be conveniently divided into two structural classes: those which adopt a compact globular fold and those which are unstructured in their monomeric state, i.e. natively unfolded proteins (table-1). A critical reading of the literature strongly suggests that some natively unfolded polypeptides populate an early oligomeric helical intermediate during amyloid formation *in vitro*. *In vivo* amyloid formation proceeds in a heterogeneous environment with the potential for interactions with membrane and with the extracellular matrix [2, 19-25]. Thus, the mechanism of amyloid formation *in vivo* may be quite different from that observed in dilute aqueous solution and characterization of the mechanism of amyloid formation in heterogeneous environments is an important goal. Again, the available evidence points to a key role for helical intermediates in at least some systems. In the remainder of the article we first provide a case by case discussion of the evidence indicating the presence of helical intermediates in amyloid formation in homogenous solution. We then turn to describing amyloid formation in the presence of membranes and components of the extracellular matrix and follow this by a short discussion of the available experimental methods for assessing the secondary structure of intermediate species. This is a major technical problem since many of the spectroscopic techniques which are routinely applied to soluble proteins are either not applicable to amyloid assembly or are much harder to employ and interpret.

2. Discussion

It is now widely recognized that a considerable number of proteins fail to fold to a compact globular structure under normal physiological conditions in the absence of their binding partners. Included among these are a number of polypeptides which form amyloid. Important examples of natively unfolded polypeptides which form amyloid *in vivo* include the Aβ peptide of Alzheimer's disease; Atrial Natriuretic Factor (ANF), which forms artial

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amyloid; Calcitonin; pro-Calcitonin, the protein component of thyroid amyloid; Islet Amyloid Polypeptide (IAPP, amylin), which is responsible for amyloid formation in type 2 diabetes; processing intermediates of pro-IAPP; α-Synuclein, responsible for Lewy body formation in Parkinson's disease and the Medin polypeptide, which is involved in Aortic medial amyloidosis (table-1). The obvious importance of Alzheimer's disease, Parkinson's disease and type 2 diabetes has stimulated considerable current interest in the mechanism of amyloid formation by natively unfolded polypeptides.

One of the more intriguing observations made with Aβ is that helical intermediates play a role in the aggregation process [26, 27]. Helix formation has been studied most extensively for the AB peptide, at least in terms of aggregation in homogenous solution. A number of lines of evidence support a role for the formation of a helical intermediate and the data is consistent with the intermediate being on pathway. Teplow and co-workers used CD to show that a transient increase in helicity is observed immediately before the appearance of β–structure suggesting that the helical intermediate is a precursor to β–sheet formation. Studies of 18 variants of Aβ showed that the intermediate is populated in each case and that the kinetics of formation of the intermediate is correlated with the time course of the appearance of fibrils [26, 27]. The mutational studies are supported by solvent perturbation experiments which examined the effects of co-solvents that are known to promote the formation of helical structure, e.g. fluorinated alcohols such as hexa-fluoro iso-propanol (HFIP) and tri-fluoro ethanol (TFE).

Helical intermediates have also been observed during amyloid formation by several designed proteins and peptides, as well as in the fibrillization of insulin [28, 29]. *In vivo*, the assembly of silk into its cross-β structure is known to proceed via a helical intermediate [30]. Time dependent studies of the *in vitro* aggregation of IAPP also suggest a transient increase in helicity, as judged by CD, before the formation of the final β-sheet structure although the data is less unambiguous than in the case of Aβ and the authors did not explicitly consider helical intermediates [31]. HFIP is commonly used to solubilze IAPP for biophysical studies and kinetic assays normally contain low levels of HFIP. HFIP is known to promote α-helical formation and is even more effective than TFE in doing so. Thus it is of interest to examine the effect of varying the HFIP concentration upon amyloid formation by IAPP. The rate of amyloid formation by IAPP is strongly dependent upon co-solvent concentration and increases as the volume fraction of HFIP increases, at least up to a point [16]. Again this is consistent with the notion that helix formation plays an important role in fibril formation. It is also interesting to consider recent high resolution NMR studies of variants of IAPP that do not form amyloid *in vivo* or *in vitro*. In particular, rat IAPP is not amyloidogenic yet differs from the highly aggregation prone human IAPP polypeptide at only 6 of 37 positions. Rat IAPP does not fold to a compact native state but residues within the segment encompassing positions 5 through 22 appears to have a modest tendency to transiently populate the helical region of the ϕ ψ map as judged by CD and NMR studies, and by analogy to the related polypeptide CGRP [32, 33]. Taken together the body of evidence is consistent with IAPP populating a helical intermediate during its self assembly to form amyloid. This is clearly an area deserving of more investigation.

Calcitonin is another polypeptide hormone which readily forms amyloid. Pro- calcitonin forms amyloid *in vivo* while the mature form of the hormone has a strong tendency to aggregate *in vitro* and form amyloid. We are not aware of any studies which have directly searched for helical intermediates during amyloid formation by Calcitonin but it is interesting to note that, while it is natively unfolded in aqueous solution, it also has some propensity to transiently populate helical structure in the monomeric state. Taken together; the body of work outlined above is highly suggestive that helical intermediates play a general role in amyloid formation by natively unfolded polypeptides. Helical intermediates

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have also been shown to play a role in the aberrant aggregation of the natively unfolded tau protein under certain conditions [34].

The studies described above all involve biophysical measurements in dilute homogenous solution. *In vivo* amyloid formation can take place in a highly heterogeneous environment potentially involving interactions with membranes and/or components of the extracellular matrix. Indeed membrane peptide interactions have been proposed to play a key role in amyloid formation in some systems while sulfated proteoglycans are found associated with *in vivo* amyloid deposits. *In vitro* biophysical studies with well defined model membrane systems or with components of the extracellular matrix clearly demonstrate that amyloidogenic polypeptides can readily adopt helical structure when bound to surfaces [35-44]. This should hardily be surprising because there is a rich literature on the induction of helical structure when amphiphilic or hydrophobic peptides bind to surfaces. Perhaps the two best characterized examples of the role of polypeptide membrane interactions in amyloid formation *in vitro* are those involving IAPP and α-synuclein [35-38, 40-43, 45]. Interaction with model membranes significantly enhances the rate of amyloid formation by IAPP and several groups have independently shown that IAPP undergoes a transition from its natively disordered monomeric state to a α-helical structure when its binds to membranes [35-38, 45]. The ability to adopt α-helical structure upon interactions with membranes appears to be conserved in IAPP molecules from different species [36]. In the systems studied, efficient peptide membrane interactions require the presence of anionic lipids. Experiments have been conducted with a range of lipid compositions, some of which are not within the physiologically range, but the basic principal that interactions with membranes induces helical structure *in vitro* is robust, weather or not they play a key role *in vivo* remains to be determined.

A recent EPR study of a large set of spin-labeled analogs of IAPP which lacked the Cys-2 Cys-7 disulfide bond has provided important information about the conformation of IAPP on the membrane [38]. The line width of the EPR signal of a nitroxide spin label is sensitive to molecular motion and can thus be used to define which regions of the peptide are ordered and which are flexible. The EPR method also provides information about the extent of burial of the nitroxide. EPR studies involve attachment of a nitroxide spin label to a Cys which has been introduced by mutation. The net effect of adding a spin label thus represents a significant perturbation, i. e. a non-conservative mutation. Hence it is safest to view the results of such studies semi- quantitatively and to bear in mind that spin labels might perturb oligomeric species or their rate of formation. An additional complication with IAPP is that it contains a disulfide bride between residues two and seven and the spin labeling studies have used variants in which the naturally occurring, (and highly conserved) Cys residues are mutated. None the less EPR studies can offer useful information and the technique is one of only a few that can provide residue specific information and the conclusions reached for IAPP membrane interactions are consistent with those derived from other methods. In the IAPP membrane study a model was proposed in which residues 9 through 22 adopt helix structure while the N and C termini are much less ordered. The model is attractive since the helical region is similar to the region which has the highest propensity to sample helical structure in the monomeric state and because it provides a conceptual frame work with which to envisage how a membrane bound helical intermediate promotes the development of β-sheet structure. Several effects come into play, association with the membrane will lead to a high local concentration of IAPP and membrane bound IAPP monomers will collide via diffusion in two rather than in three dimensions. In the model the C-terminal region of IAPP is not interacting with membranes and is much more flexible. This portion of IAPP is highly amyloidogenic thus the association of the helical regions will lead to a high local concentration of a very amyloidogenic region of the molecule [46]. Note that the model also argues that formation of helical structure could inhibit amyloid formation if the helices were

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overly stabilized since the system would be trapped in a non-amyloid conformation. The exact balance will depend on the level of stabilization of helical structure. Experiments with IAPP and certain model membranes systems have provided evidence for such effects [35, 38].

A large body of work from a number of different laboratories has shown that α-synuclein adopts partial helical structure when bound to model membranes. However the effects of protein membrane interactions on the rate of aggregation are not clear and there are conflicting reports in the literature with some workers indicating that membrane protein interactions inhibit amyloid formation while others present evidence that they enhance it [42, 43]. One possibility is that the differing results may be due to differences in the stability of the membrane bound helical structure between the studies. Recent work on another natively unfolded protein, Medin, has shown that it too populates helical structure when it interacts with membrane and that formation of the helical intermediate promotes amyloid formation [39].

Helical intermediates and amyloid formation in heterogeneous environments are not limited to membrane polypeptide interactions. For example, recent work has considered the potential role of pro-IAPP processing intermediates in amyloid formation in type 2 diabetes [47, 48]. The studies were motivated by the fact that partially processed pro-IAPP is found in amyloid deposits *in vivo*. One model postulates that interactions between the processing intermediate and sulfated proteoglycans of the extracellular matrix promotes amyloid formation [47]. *In vitro* biophysical studies have shown that such interactions do indeed efficiently promote amyloid formation by the proIAPP intermediate and furthermore have provided evidence that the process occurs via formation of a helical intermediate [44].

Experimental studies of helical intermediates have largely relied upon the use of CD to follow secondary structure formation. CD spectroscopy is very sensitive to the presence of helical structure but it can be difficult to quantitatively deconvolve CD spectra to deduce relative populations of secondary structure, particularly if light scattering becomes a problem. A second complicating factor is that the CD signal of α-helices is sensitive to the length of the helical segment and the rotational strength, (intensity), of the helical bands can be much weaker for short helices. In principle NMR does not suffer from this difficulty, however high resolution NMR studies of aggregating systems are extremely challenging and the time course of aggregation is almost always too rapid to allow the power of modern multidimensional NMR to be applied. Conventional FTIR of unlabeled peptides can be used to follow formation of helical intermediates but the helical region of amide-I band overlaps with the random coil region, making deconvolution difficult. The EPR studies briefly highlighted above allow helical structure to be detected in membrane bound systems but require the use of bulky spin labels linked to engineered Cys residues and are not non-perturbing. Ideally, one would like to define which regions of the polypeptide are sampling helical structure in both homogenous and heterogeneous systems. Recent advances in IR spectroscopy coupled with specific isotopic labeling offer the exciting prospect of being able to obtain site specific structural information with high intrinsic time resolution in a non-perturbing manner [49-52]. 2DIR with isotopic labeling will be particularly important here since it combines the virtues of good resolution while retaining the structural information. Labeling changes the reduced mass and shifts the frequency of the labeled CO oscillator. In a conventional FTIR experiment this would be lead to site specific resolution but much of the structural information can be lost because coupling with the unlabeled CO oscillators is reduced. The 2DIR method allows the structural information to be detected via cross peaks between the labeled site and the unlabeled band and retains the intrinsic high resolution due to labeling. 2DIR studies of amyloid formation are still in their infancy but exciting results have already been obtained and multiple labeling schemes have been developed which allow

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the delineation of helical regions and the method can be applied to peptide membrane systems [49-52].

3. Conclusions

How could formation of helical structure promote the conversion from a poorly structured ensemble to a partially ordered β-sheet rich structure? A number of amyloidogenic polypeptides have a propensity to transiently populate helical structure, which could be further stabilized by peptide-peptide or peptide-membrane interactions. Helix formation and self-association are well known to be linked in many systems; classic examples include naturally occurring coiled coils and other oligomerization domains, peptides with a tendency to form amphiphillic helices, and numerous designed systems. In homogenous solution initial formation of oligomers would, in this model, be driven by the thermodynamic linkage between helix formation and association. (figure-1). It is unlikely that helical structure would extend through out the molecule and there is good indirect evidence that it does not in the systems studied. Thus, helix mediated association would lead to a high local concentration of an aggregation prone sequence which in turn would promote intermolecular β-sheet formation. This model immediately suggests a potential strategy for inhibiting amyloid formation. Molecules which contain a recognition domain that can associate with the helical region but prevent convection into β-sheet structure should stop the assembly process. This is reminiscent of classic dominate negative inhibitors of certain transcript factors. Inhibitors of amyloid formation by IAPP have been designed using this strategy, although their exact mode of action remains to be determined. [53]. The model outlined in figure-1 implies that oligomer helical intermediates are on pathway but it is possible that helix intermediates could represent off pathway intermediates. We also wish to stress that we are not implying that helical intermediates are the toxic species. That is a question which cannot be addressed from any of the available data. Instead we are proposing that they play a role in the initial oligonmerization process and promote early β-sheet structures.

In membrane catalyzed systems a mechanism similar to the one proposed for IAPP membrane interactions could be generally operative. It is important to appreciate that helical intermediates do not always have to lead to an increase in the rate of amyloid formation. If the helical intermediate were too stable it could actually decrease the rate of amyloid formation since it would represent a kinetic trap. Such effects appear to be operative in IAPP membrane systems when the content of anionic lipids is high [35, 38].

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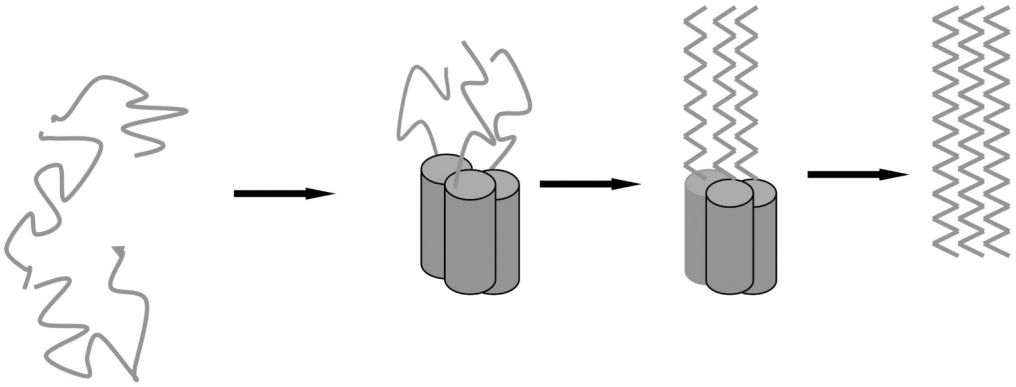

Figure-1.

A highly schematic diagram of one how α-helical intermediates might promote β-structure. α-helices are depicted as cylinders, β-sheet as zig zagging lines and coil regions as curved segments. Initial polypeptide association is driven by the thermodynamic linkage between helix formation and self association (step-1). This generates a high local concentration of a segment of the polypeptide chain which has a high amyloidogenic propensity which leads to initiation of β-structure. The β-structure propagates leading to β-sheet rich assemblies. We stress that the diagram is schematic and is not meant to imply a specific pathway for assembly for any particular system. In particular a range of oligomeric species could be formed. A trimer is depicted here. The diagram invokes a sequential zipping together of the β-strands and unzipping of the helices but this is simply meant to be illustrative and a diversity of pathways is likely. It is also possible that helical intermediates could represents off pathway species and not the on pathway intermediates depicted here.

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Table-1
Natively unfolded polypeptides which form amyloid *in vivo*

Polypeptide	Disease or Condition
$A\beta_{1-40}$ and $A\beta_{1-42}$	Alzheimer's' disease
α-Synuclein	Parkinson's disease
Medin	Amyloid deposits in the medial layer of arteries
Pro-Calcitonin	Medullary carcinoma of the thyroid
Islet Amyloid Polypeptide (IAPP, Amylin)	Type 2 Diabetes
Pro-IAPP processing intermediates[a]	Type 2 Diabetes
Glucagon	Glucagon amyloid-like fibrils: Non-toxic
Atrial Natriuretic Factor (ANF)	Artial amyloid

[a] pro-IAPP processing intermediates are thought to play a role in the *in vivo* generation of pancreatic amyloid and there is immunological evidence that they are found in islet amyloid deposits together with mature IAPP.