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Detection of helical intermediates during amyloid formation by intrinsically disordered polypeptides and proteins

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Summary

Amyloid formation and aberrant protein aggregation are hallmarks of more than 30 different human diseases. The proteins that form amyloid can be divided into two structural classes: those that form compact, well-ordered, globular structures in their unaggregated state and those which are intrinsically disordered in their unaggregated states. The latter include the Aβ peptide of Alzheimer's disease, Islet amyloid polypeptide (IAPP, Amylin) associated with type- diabetes and α-synuclein which is linked to Parkinson's disease. Work in the last ten years has highlighted the potential role of pre-amyloid intermediates in cytotoxicity and has focused attention on their properties. A number of intrinsically disordered proteins appear to form helical intermediates during amyloid formation. We discuss the spectroscopic methods employed to detect and characterize helical intermediates in solution and in membrane catalyzed amyloid formation, with the emphasis on the application of circular dichroism (CD). IAPP is used as an example, but the methods are generally applicable.

Keywords

Amyloid; Aβ Islet amyloid polypeptide; Amylin; Helical intermediate; Oligomer; CD

1. Introduction

Amyloidoses are protein aggregation diseases normally soluble, functional polypeptides and proteins self-assemble into partially ordered insoluble amyloid fibrils that deposit in tissues and organs. More than 30 different amyloidogenic polypeptides or proteins are associated with human disorders, including systematic amyloidosis, neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease, and type 2 diabetes (T2D) (1–4). Amyloid

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fibrils share common structural features even though there is no sequence homology among the proteins that form amyloid *in vivo*; theyare long, unbranched and rich in β-sheet structure. The β-strands run perpendicular to the long axis of the fibril with the hydrogen bonds aligned along the fibril axis. This cross-β conformation is common to all amyloids characterized to date.

Amyloidogenic proteins can be divided into two structural classes: those that form compact globular structures in their unaggregated state and those that are intrinsically disordered in their unaggregated states. Examples of intrinsically disordered proteins (IDPs) that form amyloid include islet amyloid polypeptide (IAPP, amylin) which is associated with pancreatic islet amyloidosis in T2D; the Aβ peptide of Alzheimer's disease and α-synuclein which is involved in Parkinson disease. There is considerable interest in characterizing pre-amyloid intermediates as they are now considered to be the most toxic species in a variety of amyloid deposition diseases. A number of IDP's that form amyloid appear to do so via the formation of an α-helical intermediate (5).

A range of spectroscopic methods are available for detecting and characterizing helical intermediates. Circular Dichorism (CD) and NMR are the most widely applied to amyloidogenic proteins. Intrinsic fluorescent probes that rely on the use of fluorescent amino acids together with a residue that and those that quenches fluorescence upon formation of an α-helical conformation have been applied to protein folding studies, and should be applicable to studies of amyloid formation (6). Infrared spectroscopy is generally less sensitive to α-helical structure and often requires concentrated samples, although two dimensional IR (2DIR) methods are being developing that extend the range of traditional IR and allow site specific detection of secondary structure during amyloid formation (7). These advanced techniques, which have largely been used to study the formation of β-sheet structure, now offer the prospect of defining the location of helices in proteins when used in conjunction with site-specific isotopically labeled samples. NMR is the method of choice for defining the secondary structure of soluble proteins in solution Both NMR and IR require more effort and higher protein concentrations than CD, and the instrumentation necessary for 2D IR measurements is currently expensive and limited to a small number of laboratories. Detection of intermediates by NMR requires that the species be trapped for a relatively long time at a suitable concentration in order to facilitate the collection of multidimensional NMR spectra. Here we focus on CD methodology as it is probably the most generally accessible technique and does not require isotopic labeling.

Islet amyloid polypeptide (IAPP, or amylin) is used as a model system example, but the approaches are general and can be applied to the study of other proteins, The choice of buffer, pH, added salts, etc. depend on the particular protein of interest.

In its physiologically functional native state, IAPP, a 37-residue post-translationally modified polypeptide hormone, is secreted from pancreatic β-cells along with insulin, and plays a role in regulating adiposity and metabolism (8–10). In T2D, the polypeptide forms amyloid by a process that is toxic to pancreatic islet β-cells and deposits as plaques in the islets (4,11–14).

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IAPP is a hydrophobic polypeptide and is cationic at physiological pH; as expected, it interacts with anionic membranes. Interactions of IAPP with model membranes containing a significant portion of anionic lipids have been widely studied. Anionic lipid vesicles, monolayers, and supported bilayers all accelerate amyloid formation by IAPP and there is good evidence that IAPP forms α-helical intermediates on model membrane surfaces (15). The relationship between reductionist *in vitro* studies with model membranes and the situation *in vivo* is ambiguous, none-the-less, considerable attention is being paid to membrane catalyzed amyloid formation (16). We also describe CD studies of IAPP/ membrane interactions in this chapter. The anionic lipid composition in the most common model membrane systems used typically ranges from 20 to 50 mole percent. In the protocol described here, we use a model membrane with 25 mole percent anionic lipids. The methods are not limited to a specific lipid composition and can be applied to other symmetric vesicles.

2. Materials

General: Deionized water and the highest grade reagents should be used. All MSDS data sheets should be carefully studied before using any reagents or solvents and appropriate waste disposal regulations should be followed. Appropriate personal protective equipments (including goggles) should be worn.

1. IAPP is usually prepared by solid phase peptide synthesis (SPPS) since the peptide is toxic to many cell lines and contains post-translation modification (C-terminally amidated) that are not generated by most expression systems. IAPP can be synthesized via 9-fluornylmethoxycarbonyl (Fmoc) chemistry or *tert*-Butyl carbamate (t-Boc) methods (*see* Note 1). The Alzheimer's Aβ peptide is also typically prepared by SPPS, usually by t-Boc approaches. IAPP is purified by reverse-phase high pressure liquid chromatography (HPLC) using a C18 preparative column (*see* Note 2). The identity of the purified peptide should be confirmed by mass spectrometry, even for commercially purchased samples. IAPP contains multiple Asn residues and can undergo spontaneous deamidation in which Asn residues are converted into mixtures of L-Asp, D-Asp, L-iso-Asp and D-iso-Asp (17). It is important to check the integrity of the polypeptide before commencing experiments. The peptide should be stored as a dry powder at −20 °C or −80 °C in a sealed container with desiccant. Care should be used when removing samples from the freezer. The peptide, still in its container, should be placed in a desiccator and allowed to warm to room temperature before the container is opened. This will help to minimize undesired absorption of water.

[1]The IAPP samples used in this example were prepared using Fmoc chemistry. Fmoc protected pseudoproline dipeptide derivatives were incorporated to facilitate the synthesis. The Cys-2 and Cys-7 disulfide bond in IAPP was generated via oxidation by DMSO in the present example (23).

[2]HCl is the preferred ion-pairing agent instead of TFA for HPLC purification of IAPP, even though TFA usually yields better resolution and peak separation. The reason is that excess TFA can cause problems with toxicity in cellular assays and TFA influences the aggregation kinetics of some peptides derived from IAPP (24).

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2. Lipids: 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl sn glycerol-3phospho-(1'-rac-glycerol) (DOPG) are used in the example described here and were obtained from Avanti Polar Lipids. Lipid stock solutions were prepared in chloroform and stored at −80°C (*see* Note 3 and 4).

3. General considerations for the choice of buffers for CD studies: The highest grade analytical reagents should be used. A key concern with CD studies is the necessity to avoid significant background absorbance. CD is a difference technique with the signal representing the difference in absorbance of right vs. left circular polarized light. The differential absorbance is small, typically 10-4 to 10-6 absorbance units (A.U.) for a bio-molecule that contributes an optical density of 1.0. This means that less than 0.1 % of the total absorbance signal must be measured accurately and precisely. The practical consequence is that a small difference needs to be measured precisely and a significant background absorbance from buffer or salts can be a compilation. For example, a 10 mm solution of NaCl will contribute an absorbance of greater than 0.5 A.U. at 190 nm in a 0.1 cm cell. Protein CD spectra are often recorded using NaF instead of NaCl since the fluoride salt has a smaller absorbance. However, the rate of IAPP amyloid formation is sensitive to the choice of the anion in solution (18), even at moderate salt concentrations. Control experiments should be conducted if one changes buffers or salts to ensure that the rate of amyloid formation is not significantly impacted. The same considerations hold for other amyloidogenic proteins, and peptides. Some common buffers also have significant background absorbance in the far UV and are best avoided, if possible. Tricine, Hepes and to a lesser extent, Pipes, all absorb in the far UV. Phosphate, Borate, Tris, Mes and Caccodylate absorb less. It is important to use a buffer whose pH is insensitive to temeprature if temeprature dependent studies are to be conducted.

3. Methods

All sample preparation procedures can be carried out at room temperature unless otherwise specified.

3.1. Preparation of proteins and polypeptides

Working with amyloidogenic proteins is challenging and there are contradictory reports in the literature on the cytotoxic and conformational properties of these polypeptides. The confusion is likely, in large part duet to differences in the methods used to solublize and study the polypeptides. A range of methods, have been developed to prepare amyloidogenic polypeptides and proteins in, hopefully, initially monomeric states. However low order oligomers are often detected as soon as the polypeptide is dissolved, meaning that it is very hard to be certain that one is starting a measurement from a monomeric state. The specific protocol used to prepare samples depends on the protein being investigated. The reader is

[3]Lipids should be stored dry at −80 °C, if long-term storage is required, to prevent potential decomposition.
[4]Glassware rather than plastic ware, should be used when handling organic solvents such as chloroform.

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referred to the literature for examples of protocols used with other proteins. The example described here is applicable to IAPP.

1. Dry IAPP is dissolved in 100% HFIP. The peptide stock solution is incubated for several hours until the solution is clear and then filtered through a 0.22 um filter (*see* Note 5).

2. Trace amounts of co-solvents can influence the properties of IAPP and other polypeptides and effect their rate of amyloid formation. Thus aliquots of the IAPP/HFIP stock solution are freeze dried to remove organic solvents before the samples are used in experiments. The freeze drying step should be carried out at the coldest temperature under the strongest possible vacuum for a duration of at least 12 to 24 hrs to insure the removal of residual organic solvents.

3. To initiate the the experiment, the dry peptide is re-dissolved in an appropriate CD buffer (see section **2.3**.) with an appropriate choice of salt at the desired pH (*see* Note 6) and CD spectra are recorded as a function of time. Amyloid formation by human IAPP (h-IAPP) is strongly pH dependent and is faster when the side chain of the single His residue and the N-terminus are neutral. The rate of hIAPP amyloid formation also depends upon on ionic strength and, as noted above, on the choice of the anion.

3.2. Preparation of LUVs for peptide membrane studies

This protocol is described in more detail in the accompanying chapter in this volume on IAPP model membrane interactions. The membrane used for this example contains 25% anionic lipids by mole percent and is comprised of a mixture of DOPC and DOPG (*see* Note 7).

1. Chloroform stock solutions of DOPC and DOPG are added to a round-bottom glass flask at a 3:1 molar ratio (*see* Note 8). Organic solvent is removed by evaporation using a stream of nitrogen gas to generate a film. The lipid film is further dried overnight under a vacuum in order to remove any residual organic solvent.

2. The lipid film is re-dissolved in the desired buffer and agitated for one hour using stirring or mild shaking (*see* Note 9).

3. After rehydration, the lipid suspension is subjected to 10 freeze-thaw cycles and then extruded 15 times through 100 nm pore size filters (Whatman, GE) (*see* Note 10 and 11).

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[5]Filtration is recommended to remove large aggregates.

[6]The concentration of the peptide solution should be tested to check for any loss during filtration. The concentration can be estimated using the absorbance at 280 nm. hIAPP lacks Trp, but contains two Phe and one Tyr residues. Peptide concentrations can also be determined by quantitative amino acid analysis or by using the BCA or Bradford assays (25–26). It is important to stress that concentration should not be determined by weight. Dried synthetic peptides contain salts and residual water; this can lead to significant errors in concentration measurements by weight, which in turn impacts the interpretation and analysis of CD data.

[7]Different lipid compositions can be used, but the basic protocol is applicable to other symmetric lipid vesicles. We use a 25% anionic model membrane system as an example here, but more complicated lipid mixtures can be used.

[8]The lipids must be mixed thoroughly to obtain a homogeneous solution.

[9]During the hydration step, the lipid suspension needs to be maintained at a temperature above the highest gel-liquid crystal transition temperature of any of the mixed lipids.

4. The phospholipid concentration of the resulting LUVs can be determined using the method of Stewart (19).

3.3. CD measurements

The sensitivity of a CD measurement depends on the absorbance of the CD active molecule of interest (and thus its concentration), the spectral bandwidth used, the time constant, the step size used to scan the spectrum and the path length. The range of absorbance (from the compound of interest) for CD is typically on the order of 0.5 to 2.0 A.U. with an OD near 0.87 being optimal (Information provided by the AVIV Instruments CD Manual). Of course, the concentration of the sample is also dictated by the biophysics of the system. Too high a concentration may lead to rapid aggregation and hinder the detection of intermediates or alter the mechanism of assembly. Far UV CD spectra of human IAPP are typically recorded with a protein concentration of 5 to 40 μM. The relevant concentration for far UV CD spectra of proteins is the concentration of peptide bonds, thus different proteins require different amounts of protein. Protein and polypeptide far UV CD spectra are typically recorded in a 0.1 cm cuvette. A longer path length cell is usually used for near UV CD, but can result in too strong a background absorbance when used for far UV CD. One should always check for sample absorption to the walls of the cuvette when studying a new protein or when studying a protein under new conditions. The reader is referenced to the manual for his/her specific type of CD instrument for suggestions on the optimal instrumental parameters to use. Our laboratory typically records CD spectra of 5 to 40 μM samples of human IAPP in low salt using Tris buffer with a 1 second time constant and a 1 nm bandwidth over the range of 260 nm to as low as possible (typically 195 to 185 nm).

3.4. Analysis of CD spectra and detection of α-helical intermediates

Fortunately, α-helices are relatively regular elements of secondary structure and have fewer variations in their basic geometry than do β-sheets. Thus, it is reasonable to speak of the CD spectrum of a typical α-helix. In addition, the structural features that can lead to variations in α-helical CD spectra have been well studied (20). There are essentially two methods for estimating the helical content of a protein from the CD spectrum. One can deconvolve (fit) the spectrum using a number of freely available programs. Most are accessible on the web (21). There are two important considerations when doing so. First, one should strive to record the far UV CD spectrum to as low a wavelength as possible. Truncation of the spectra at too high a wavelength can lead to ambiguity in decovolutions. Second, an accurate measurement of the polypeptide concentration is helpful. The second method for deducing the amount of helical structure relies on the signal intensity, the mean residue ellipticity, at 222 nm, $[\theta]_{222}$. Of course, a relaible estimate requires that other features do not contribute significantly to the CD signal at this wavelength. The signal for a fully helical peptide, $[\theta]_{H}$, and for a fully unfolded peptide, $[\theta]_{C}$, are needed. Both the fully helical intesity and the coil

[10]Extrusion is used to form large unilamellar vesicles and should be performed at a temperature above the Tc of the mixed lipids. The pore size of the filter used depends on the required size of the lipid vesicles (usually LUVs are in the range of 200 to 1000 nm). Small unilamellar vesicles, (diameter 15 to 50 nm) can be prepared by sonication.

[11]LUVs can be stable for up to several days after preparation, however, it is recommended that vesicles be prepared fresh on the day of experiments. The uniformity of the lipid vesicles can be checked by cryo-electron microscopy and by light scattering.

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intensity can be temeprature dependent and this can be taken into account using empirical relationships (22). One popular set is:

$$[\theta]_{\mathrm{H}}=-40,000 \times (1-2.5/n)+100 \times T \quad \text{(eq 1)}$$

$$[\theta]_{\mathrm{C}}=640-45 \times T \quad \text{(eq 2)}$$

Where n is the number of the residues in the peptide, and T is the temperature (°C). A key aspect of the analysis is that one must know the peptide concentration as accurately as possible. Errors in concentration determination translate directly into errors in mean residue ellipticity and directly to errors in the estimated α-helical content. It is important to stress that determining concentration by weight is very imprecise and prone to error, and is not adequate for a quantitative analysis of spectral intensities.

The estimated amount of helical structure present in a partially structured ensemble deduced by CD can differ from that deduced using NMR secondary chemical shifts. There is no inherent contradiction with the two methods, rather they offer complimentary information. NMR secondary shifts are local in origin and provide information on the fraction of the time a particular residue populates the helical region of the Ramachandran plot. The CD spectra of helical peptides are length dependent and a very short helix, or an isolated residue populating ϕ ψ helical angles, will not give rise to significant intensity at 222nm.

As a final note we stress that the observation of an isodichrocic point (isobestic point) in a set of CD spectra does *not* prove that the transition being monitored is two-state. An isobestic point is a necessary, but not a sufficient condition for a two-state transition. The misinterpretation of isobestic points in CD spectra is probably one of the most common errors in the literature.

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References

1. Selkoe DJ. Cell biology of protein misfolding: The examples of Alzheimer's and Parkinson's diseases. Nat Cell Biol. 2004; 6:1054–1061. [PubMed: 15516999]
2. Chiti F, Dobson CM. Protein misfolding, functional amyloid, and human disease. Annu Rev Biochem. 2006; 75:333–366. [PubMed: 16756495]
3. Sipe JD. Amyloidosis. Crit Rev Clin Lab Sci. 1994; 31:325–354. [PubMed: 7888076]
4. Westermark P, Andersson A, Westermark GT. Islet amyloid polypeptide, islet amyloid, and diabetes mellitus. Physiol Rev. 2011; 91:795–826. [PubMed: 21742788]
5. Abedini A, Raleigh DP. A critical assessment of the role of helical intermediates in amyloid formation by natively unfolded proteins and polypeptides. Protein Eng Design & Select. 2009; 22:453–459.

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6. Taskent-Sezgin H, Marek P, Thomas R, Goldberg D, Chung J, Carrico I, Raleigh DP. Modulation of p-cyanophenylalanine fluorescence by amino acid sidechains and rational design of fluorescence probes of a-helix formation. Biochemistry. 2010; 49:6290–6295. [PubMed: 20565125]
7. Shim S-H, Gupta R, Ling YL, Strasfeld DB, Raleigh DP, Zanni MT. 2DIR spectroscopy defines the pathway of amyloid formation with residue specific resolution. Proc Natl Acad Sci USA. 2009; 106:6614–6619. [PubMed: 19346479]
8. Lukinius A, Wilander E, Westermark GT, Engstrom U, Westermark P. Co-localization of islet amyloid polypeptide and insulin in the beta-cell secretory granules of the human pancreatic-islets. Diabetologia. 1989; 32:240–244. [PubMed: 2668077]
9. Kahn SE, Dalessio DA, Schwartz MW, Fujimoto WY, Ensinck JW, Taborsky GJ, Porte D. Evidence of cosecretion of islet amyloid polypeptide and insulin by beta-cells. Diabetes. 1990; 39:634–638. [PubMed: 2185112]
10. Stridsberg M, Sandler S, Wilander E. Cosecretion of islet amyloid polypeptide (IAPP) and insulin from isolated rat pancreatic-islets following stimulation or inhibition of beta-cell Function. Regul Pept. 1993; 45:363–370. [PubMed: 8351401]
11. Clark A, Wells CA, Buley ID, Cruickshank JK, Vanhegan RI, Matthews DR, Cooper GJS, Holman RR, Turner RC. Islet amyloid, increased alpha-cells, reduced beta-cells and exocrine fibrosis - quantitative changes in the pancreas in type-2 diabetes. Diabetes Res Clin Ex. 1988; 9:151–159.
12. Lorenzo A, Razzaboni B, Weir GC, Yankner BA. Pancreatic-islet cell toxicity of amylin associated with type-2 diabetes-mellitus. Nature. 1994; 368:756–760. [PubMed: 8152488]
13. Konarkowska B, Aitken JF, Kistler J, Zhang S, Cooper GJ. The aggregation potential of human amylin determines its cytotoxicity towards islet beta-cells. FEBS J. 2006; 273:3614–3624. [PubMed: 16884500]
14. Cao P, Marek P, Noor H, Patsalo V, Tu LH, Wang H, Abedini A, Raleigh DP. Islet amyloid: From fundamental biophysics to mechanisms of cytotoxicity. FEBS Lett. 2013; 587:1106–1118. [PubMed: 23380070]
15. Hebda JA, Miranker AD. The interplay of catalysis and toxicity by amyloid intermediates on lipid bilayers: insights from type II diabetes. Annu Rev Biophys. 2009; 38:125–152. [PubMed: 19416063]
16. Cao P, Abedini A, Wang H, Tu LH, Zhang XX, Schmidt AM, Raleigh DP. Islet amyloid polypeptide toxicity and membrane interactions. Proc Natl Acad Sci U S A. 2013; 110:19279–19284. [PubMed: 24218607]
17. Dunkelberger EB, Buchanan LE, Marek P, Cao P, Raleigh DP, Zanni MT. Deamidation accelerates amyloid formation and alters amylin fiber structure. J Am Chem Soc. 2012; 134:12658–12667. [PubMed: 22734583]
18. Marek P, Pastalo V, Green D, Raleigh DP. Ionic strength effects on amyloid formation by amylin are a complicated interplay among Debye screening, ion selectivity, and Hofmeister effects. Biochemistry. 2012; 51:8478–8490. (2012). [PubMed: 23016872]
19. Stewart JCM. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal Biochem. 1980; 104:10–14. [PubMed: 6892980]
20. Manning MC, Woody RW. Theoretical CD studies of polypeptide helices- examination of important electronic and geometric factors. Biopolymers. 1991; 31:569–586. [PubMed: 1868170]
21. Whitmore L, Wallace BA. Protein secondary structure analyses from circular dichroism spectroscopy: methods and reference databases. Biopolymers. 2008; 89:392–400. [PubMed: 17896349]
22. Scholtz JM, Baldwin RL. Ann Rev Biophys & Biomol Struct. 1992; 21:95–118. [PubMed: 1525475]
23. Abedini A, Singh G, Raleigh DP. Recovery and purification of highly aggregation-prone disulfide-containing peptides: Application to islet amyloid polypeptide. Anal Biochem. 2006; 351:181–186. [PubMed: 16406209]
24. Nilsson MR, Raleigh DP. Analysis of amylin cleavage products provides new insights into the amyloidogenic region of human amylin. J Mol Biol. 1999; 294:1375–1385. [PubMed: 10600392]

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25. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248–254. [PubMed: 942051]

26. Zor T, Selinger Z. Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. Anal Biochem. 1996; 236:302–308. [PubMed: 8660509]