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The Fluorescent Amino Acid *p*-Cyanophenylalanine Provides an Intrinsic Probe of Amyloid Formation

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Amyloid formation has been implicated in more than fifteen different human diseases including Alzheimer's disease, Parkinson's disease, prion-based diseases and type 2 diabetes.[1,2] The kinetics of amyloid formation are complex, typically consisting of a lag phase during which little fibrillar material is produced followed by a rapid growth phase. Characterization of the kinetics of amyloid formation, and the nature of any intermediates which are formed have emerged as critical topics in the field since there is growing evidence that pre-fibrillar intermediate structures may be the toxic species.[3] Unfortunately, a limited set of low resolution spectroscopic methods can be applied to study the kinetics of amyloid formation and residue specific information is generally not obtainable.

Amyloid formation, *in vitro*, is traditionally followed using fluorescence detected thioflavin binding experiments. The fluorescence of the dye significantly increases upon binding to the amyloid fibril. The assay is simple to execute, however, it does suffer from some noticeable drawbacks.[4] First, the exact mechanism for the fluorescence enhancement is not completely understood, hence, it is not completely clear what the dye binding probes. Second, the dye does not bind to pre-fibrillar intermediates and thus cannot be used to follow their formation. A third, extremely important, but somewhat subtle issue involves the study of inhibitors. Some compounds can bind to amyloid fibrils, displacing bound thioflavin-T without inhibiting amyloid formation.[5] In these cases thioflavin-T assays lead to the incorrect conclusion that the compound is an amyloid inhibitor. Fourth, the dye is an extrinsic probe and there is always the risk that the kinetics of assembly could be affected since the assay is conducted by adding the dye to the peptide solution and it binds to the fibrils as they are forming.

In principle, intrinsic protein fluorescence could be used to follow amyloid formation since Trp fluorescence is sensitive to the local environment. However, a surprising number of important amyloidogenic polypeptides lack Trp including Aβ, α-synuclein and IAPP (amylin), the causative agents of amyloid formation in Alzheimer's disease, Parkinson's disease and type-2 diabetes respectively. Furthermore, the addition of Trp by mutagenesis often represents a non-conserved mutation. Tyr fluorescence might be useful, but it is less sensitive than Trp fluorescence and its interpretation is much less straightforward. It would clearly be desirable to have access to another fluorescent amino acid that could be used as a probe of amyloid formation.

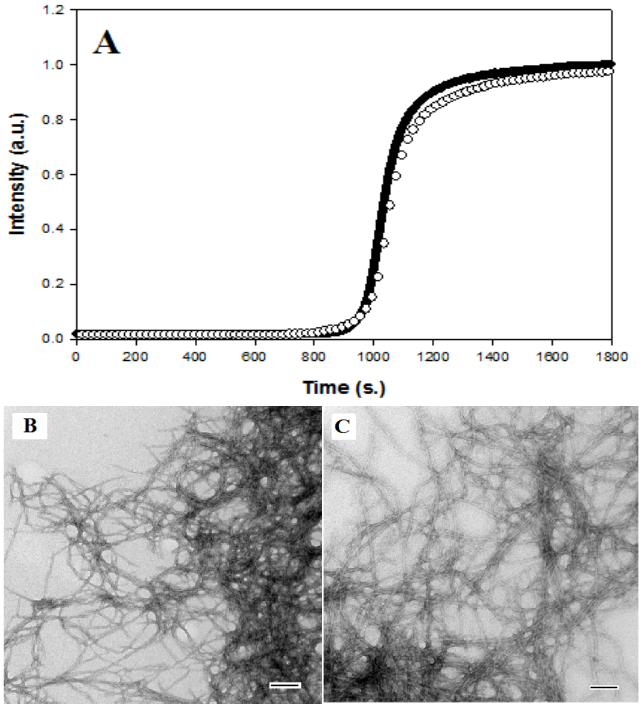

Figure 1. (A) Comparison of the time course for thioflavin-T fluorescence for wild-type hIAPP (●) and hIAPP-Y37$F_{C\equiv N}$ (○). The data is normalized on a scale from 0 to 1.0. (B) TEM images of the fibrils formed by wild-type human IAPP and (C) the Y37$F_{C\equiv N}$ variant. Scale bar represents 100 nm. Experiments were performed in 2% HFIP, 20 mM Tris-HCl, pH 7.4.

An ideal amino acid analog would exhibit a large, easily interpretable change in fluorescence during the process of amyloid formation, but represent only a small perturbation on the structure and hydrophobicity of one or more of the twenty genetically encoded residues, allowing for conservative substitution. *p*-Cyanophenyalanine (*p*-cyanoPhe) appears to meet all of these requirements.[6] Its fluorescence quantum yield is very sensitive to solvent interactions and is decreased significantly in a hydrophobic environment compared to its value in water, making it a sensitive probe of the local environment. Importantly, it has a blue-shifted absorption band allowing its fluorescence to be selectively excited in the presence of Tyr or Trp. The cyano group is a hydrogen bond acceptor but it has the very desirable feature that it is readily accommodated in the hydrophobic core of proteins since its polarity is intermediate between that of an amide and a methylene.[6] It is also considerably smaller than Trp, making it a very conservative replacement for Phe or Tyr.

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In the present work we demonstrate the use of *p*-cyanoPhe fluorescence to probe amyloid formation using islet amyloid polypeptide (IAPP, amylin) as a test case. IAPP is responsible for the formation of pancreatic islet amyloid in type 2 diabetes.

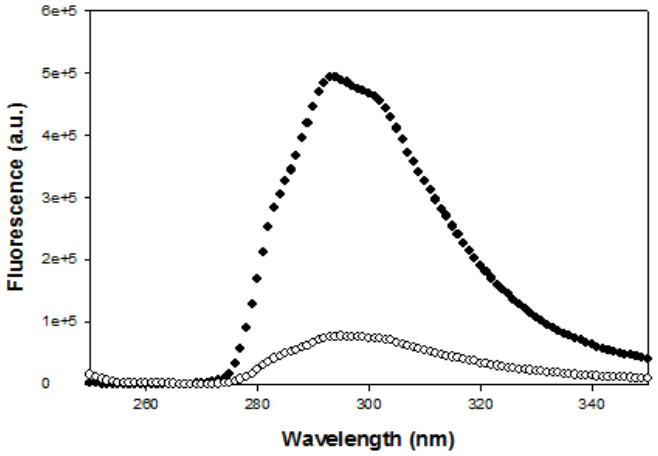

Figure 2. *p*-cyanoPhe fluorescence emission spectra of hIAPP-Y37F$_{C\equiv N}$ at the start of the fibrillization reaction (●) and at the end of the reaction (○). Experiments were performed in 2% HFIP, 20 mM Tris-HCl, pH 7.4.

Islet amyloid formation plays a role in the pathology of the disease by killing the pancreatic β-cells and contributing to the loss of β-cell mass and the decline in insulin secretion.[7] IAPP is 37 residues in length, contains a disulfide bond linking residues 2 and 7 and has an amidated C-terminus. It contains no Trp, but does have two Phe's at positions 15 and 23 and a single Tyr at its C-terminus. We replaced Tyr-37 with *p*-cyanoPhe. The peptide is denoted hIAPP-Y37F$_{C\equiv N}$. The sequence of the wild-type human peptide, denoted here hIAPP, is:

KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY

In vitro assays of amyloid formation often involve solubilizing the peptide of interest in a fluorinated alcohol, typically hexafluoroisopropanol (HFIP). The fibrillization reaction is initiated by diluting the stock solution into aqueous buffer. *p*-CyanoPhe fluorescence is sensitive to the hydrogen bonding properties and polarity of the solvent, thus it is important to test whether or not this protocol significantly affects its fluorescence. We prepared a small, soluble *p*-cyanoPhe containing peptide (Gly-Phe$_{C\equiv N}$-Ala-Ala) for the control studies. The fluorescence intensity was the same in water, 2% HFIP and was very similar in 100% HFIP, indicating that there are no problems associated with standard fibrillization protocols. 4-cyanoPhe fluorescence is quenched by chloride ion and many biological buffers are made from chloride salts, thus we tested if 20 mM Tris-HCl significantly affected the fluorescence. The fluorescence intensity was reduced by approximately 30%. This does not present any significant problems since the fluorescence of 4-cyanoPhe will decrease much more if it is buried in a hydrophobic environment.

We next compared the time course of amyloid formation for wild-type and hIAPP-Y37F$_{C\equiv N}$ in order to test if the replacement of the phenolic OH group by a cyano group had a significant effect. Standard thioflavin-T kinetic assays demonstrate that the time course of fibril formation by wild-type hIAPP and hIAPP-Y37F$_{C\equiv N}$ are essentially identical. Quantitative analysis of the data shows that the t_{50} times (the time for the reaction to reach 50% of the maximum fluorescence) are virtually the same, 960±60 s. for wild-type and 1130±100 s. for hIAPP-Y37F$_{C\equiv N}$. The time of the growth phases, here defined as the time required to go from 10% to 90% of the maximum fluorescence, was 208±13 s. for wild-type and 247±12 s. for the variant. The final values of thioflavin-T fluorescence were identical. The observation of identical values of

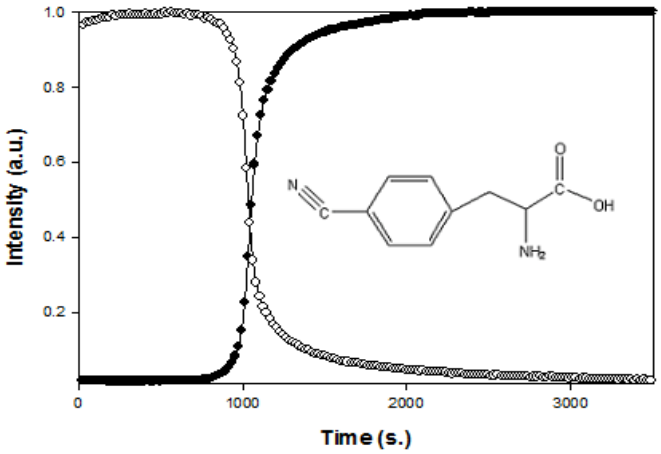

Figure 3. Comparison of the time course of thioflavin-T fluorescence (●) at 480 nm and *p*-cyanoPhe fluorescence (○) at 296 nm for hIAPP-Y37F$_{C\equiv N}$ at pH 7.4, 25°C. The data is normalized so that the total signal change is displayed on a scale of 0.0 to 1.0. The molecular representation of 4-cyanoPhe is shown as an insert. The non-normalized data is included in the supporting information.

thioflavin-T fluorescence at the end of the reaction argues that the *p*-cyanoPhe substitution does not significantly affect the morphology of the fibrils. This is confirmed by transmission electron microscopy (TEM). TEM images of hIAPP and hIAPP-Y37F$_{C\equiv N}$ are indistinguishable (Figure-1). CD spectra of hIAPP and hIAPP-Y37F$_{C\equiv N}$ are also identical (supplemental information). The kinetic, spectroscopic and TEM studies all demonstrate that the *p*-cyanoPhe substitution for Tyr is indeed very conservative.

Having confirmed that hIAPP-Y37F$_{C\equiv N}$ forms amyloid deposits that are similar to wild-type, we turned to kinetic investigations using *p*-cyanoPhe fluorescence. There is a large change in fluorescence between the soluble form of the peptide and the fibril form (Figure-2). Figure-3 compares the time course monitored using *p*-cyanoPhe fluorescence to the time course monitored using thioflavin fluorescence. The same stock solution and the same cuvette were used for both measurements. This is important because the time course of amyloid formation is sensitive to the shape of the cuvette and volume of solution used. The ability to conduct experiments under absolutely identical conditions on the same instrument is a key advantage. The plot of thioflavin fluorescence vs. time shows the characteristic sigmoidal curve observed in studies of amyloid formation. The time course of the *p*-cyanoPhe fluorescence is identical to that observed for the thioflavin-T experiment. The midpoints (t_{50}) of the two experiments are identical: 1,100±100 s. as determined by *p*-cyanoPhe fluorescence and 1130±100 s. from thioflavin-T fluorescence. Likewise, the times of the growth period determined by the two methods are the same: 248±12 s. as determined by *p*-cyanoPhe fluorescence and 247±12 s. from thioflavin-T fluorescence.

The experimental results demonstrate the utility of *p*-cyanoPhe fluorescence as a probe of IAPP fibrillization and importantly provide new insight into amyloid formation by IAPP. The data here shows that burial of the C-terminal aromatic side chain does not occur during the lag phase, but rather occurs concomitantly with fibril assembly during the growth phase. Tyr-Phe fluorescence energy transfer studies have been interpreted to indicate that hIAPP forms a collapsed conformation during the lag phase.[8] Our results demonstrate that the C-terminal residue is buried on the same time scale as fibril formation, ruling out pre-fibrillar intermediates in which the C-terminal side chain is buried. The data presented here also shows that the C-terminal side chain is partially sequestered from solvent. This offers an explanation for the apparent elevated pKa for Y37 in the fibril.[8]

A number of important amyloidogenic polypeptides contain Phe and or Tyr, but lack Trp. These include $A\beta_{1-42}$, $A\beta_{1-40}$, calcitonin, insulin and α-synuclein. Thus, *p*-cyanoPhe substitutions are expected to be a generally useful approach to probe amyloid formation especially considering that the derivative can be readily incorporated into proteins by chemical synthesis or by recombinant methods.[6,9] The IAPP analog described here should be a useful reagent for studies of fibrillization inhibitors since it avoids the problems associated with the use of extrinsic probes.[12]

Experimental Section

Wild-type IAPP, $Y37F_{C\equiv N}$ IAPP and the G-F_{CN}-A-A tetrapeptide were synthesized as described. The disulfide bond in IAPP was formed using DMSO based oxidation.[10] Fmoc-4-cyanoPhe was obtained from NovaBiochem. Peptides were purified via reverse phase HPLC and the identities confirmed by MALDI mass spectroscopy.

Thioflavin-T fluorescence experiments were performed as described. *p*-cyanoPhe fluorescence was excited at 240 nm and detected at 296 nm with slit widths of 10 nm on an Applied Photon Technologies fluorimeter. Stock solutions of IAPP in HFIP (1.58 mM) were prepared as described.[11] Fibrillization reactions were initiated by diluting the stock 50-fold into buffered (20 mM Tris-HCl, pH 7.4) aqueous solution. Final conditions were 32 μM IAPP, 20 mM Tris-HCl, 2% HFIP at pH 7.4. When present, thioflavin-T was at 32 μM.

TEM was performed at the University Microscopy Imaging Center at the State University of New York at Stony Brook. 4 μL samples from the wild-type and $Y37F_{C\equiv N}$ IAPP reaction solutions were placed on a carbon-coated 300-mesh copper grid and negatively stained with saturated uranyl acetate.

Far-UV CD experiments were performed at 25 °C on an Aviv 62A DS CD spectrophotometer. For far-UV CD wavelength scans, an aliquot from the peptide stock was diluted into 20 mM Tris-HCl buffer at pH 7.4, for a total volume of 250 μL. The final peptide concentration was 0.1 mg/mL in 20 mM Tris-HCl buffer. The spectrum is the average of three repeats in a 0.1 cm quartz cuvette and recorded over a range of 190-250 nm, at 1nm intervals with an averaging time of three seconds per scan. A background spectrum was subtracted from the collected data.

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Keywords: amyloid · biosensors · islet amyloid polypeptide · *p*-cyanophenylalanine · peptides

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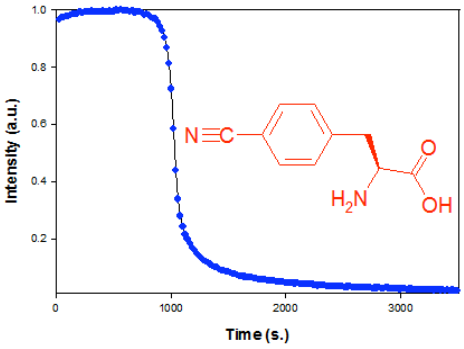
Analogs of amyloid forming polypeptides have been developed using *p*-cyanophenylalanine as a sensitive, non-invasive, intrinsic probe of amyloid formation. *p*-Cyanophenylalanine is a very conservative substitution for phenylalanine or tyrosine and its fluorescence is incredibly sensitive to the environment, making it a powerful probe of amyloid formation.



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Supporting Information

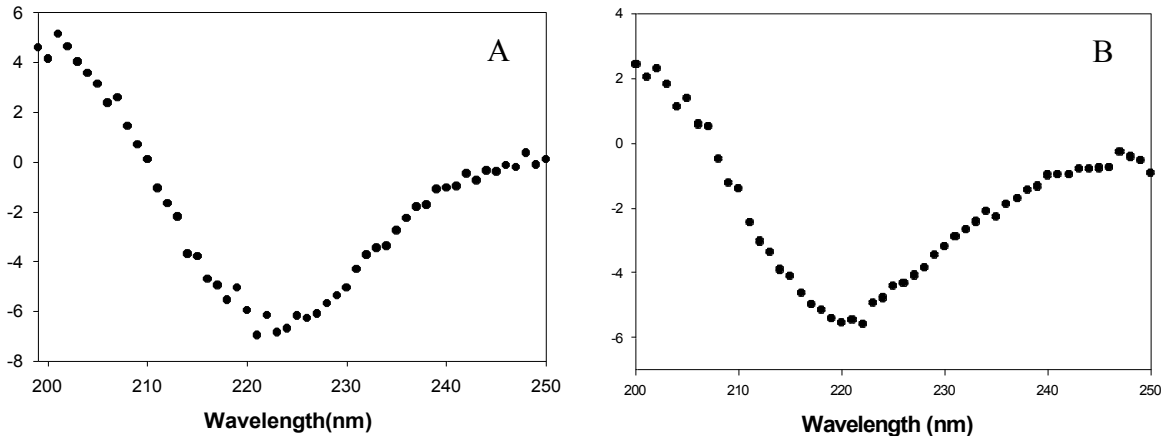

Figure S1: CD spectra of a sample of hIAPP-Y37F$_{C\equiv N}$ (A) and wild-type (B) recorded at the end of the kinetic experiment. Conditions are 32 μM peptide, 32 μM thioflavin-T, 20 mM Tris pH 7.4, 2% HFIP (v/v), 25°C. Samples were not stirred during the collection of the spectrum.