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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, and typically consist 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 that are formed have emerged as critical topics in the field since there is growing evidence that prefibrillar intermediate structures might 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 by fluorescence detection in thioflavin T-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 prefibrillar 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 and displace 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 being formed.

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 nonconserved 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. An ideal amino-acid analogue should 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, and thus allow 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; this makes it a sensitive probe of the local environment. Importantly, it has a blue-shifted absorption band, which allows 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, which makes it a very conservative replacement for either Phe or Tyr.

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. Islet amyloid formation plays a role in the pathology of the disease by killing 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 that links residues 2 and 7, and has an amidated C terminus. It does not contain Trp, but does have two Phe residues at positions 15 and 23 and a single Tyr at its C terminus. We replaced Tyr37 with *p*-cyanoPhe. The peptide is denoted hIAPP-Y37$F_{C\equiv N}$. 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 in 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; this indicates that there are no problems associated with standard fibrillization protocols. 4-CyanoPhe fluorescence is quenched by chloride ions and many biological buffers are made from chloride salts, thus we tested if Tris-HCl (20 mM) significantly affected the fluores-

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cence 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 peptide 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 demonstrated 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 $\pm$ 60) s for wild-type peptide and (1130 $\pm$ 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 $\pm$ 13) s for the wild-type peptide and (247 $\pm$ 12) s for the variant. The final values of thioflavin T fluorescence were identical. The observation of identical values of thioflavin T fluorescence at the end of the reaction suggests that the *p*-cyanoPhe substitution does not significantly affect the morphology of the fibrils. This was confirmed by transmission electron microscopy (TEM). TEM images of hIAPP and hIAPP-Y37F$_{C\equiv N}$ were indistinguishable (Figure 1). CD spectra of hIAPP and hIAPP-Y37F$_{C\equiv N}$ were also identical (see the Supporting Information). The kinetic, spectroscopic, and TEM studies all demonstrate that *p*-cyanoPhe substitution for Tyr is indeed very conservative.

Having confirmed that hIAPP-Y37F$_{C\equiv N}$ forms amyloid deposits that are similar to the wild-type peptide, 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 by using *p*-cyanoPhe fluorescence to the time course monitored by using thioflavin T 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 by using the same instrument is a key advantage. The plot of thioflavin T fluorescence versus time showed the characteristic sigmoidal curve observed in studies of amyloid formation. The time course of *p*-cyanoPhe fluorescence was identical to that observed in the thioflavin T experiment. The midpoints (t_{50}) of the two experiments were identical: (1100 $\pm$ 100) s as determined by *p*-cyanoPhe fluorescence and (1130 $\pm$ 100) s from thioflavin T fluorescence. Likewise, the times of the growth period determined by the two methods were the same: (248 $\pm$ 12) s as determined

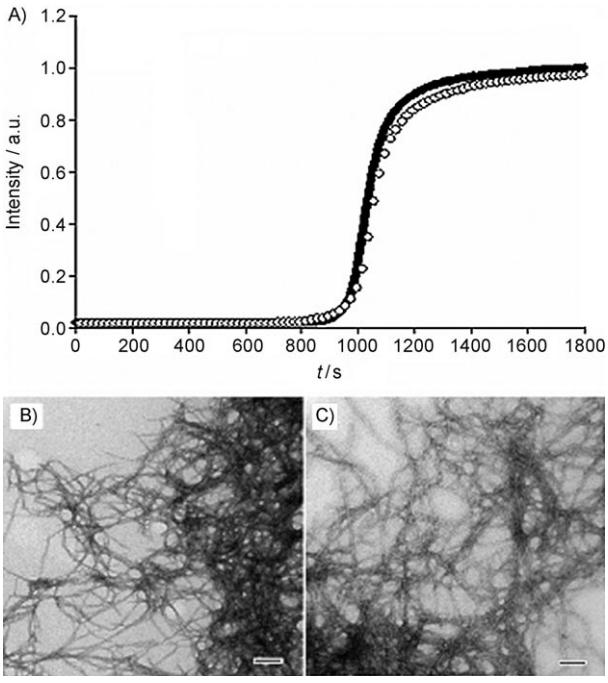

Figure 1. A) Comparison of the time course for thioflavin T fluorescence for wild-type hIAPP (●) and hIAPP-Y37F$_{C\equiv N}$ (○). The data were normalized on a scale from 0.0 to 1.0. TEM images of the fibrils formed by B) wild-type human IAPP, and C) the Y37F$_{C\equiv N}$ variant; scale bar represents 100 nm. Experiments were performed in 2% HFIP, 20 mM Tris-HCl, pH 7.4.

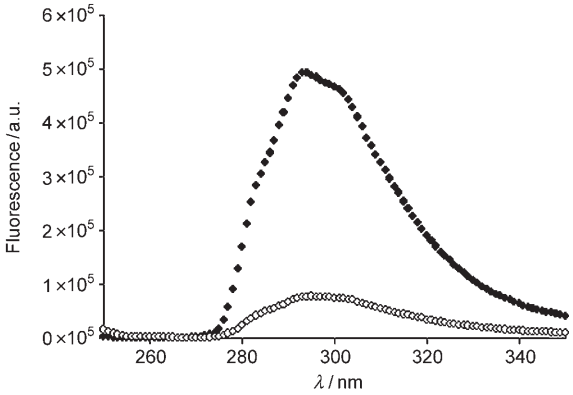

Figure 2. *p*-CyanoPhe fluorescence emission spectra of hIAPP-Y37F$_{C\equiv N}$ at the start of the fibrillization reaction (●) and at the end of the reaction (○). Experiments were performed in 2% HFIP, 20 mM Tris-HCl, pH 7.4.

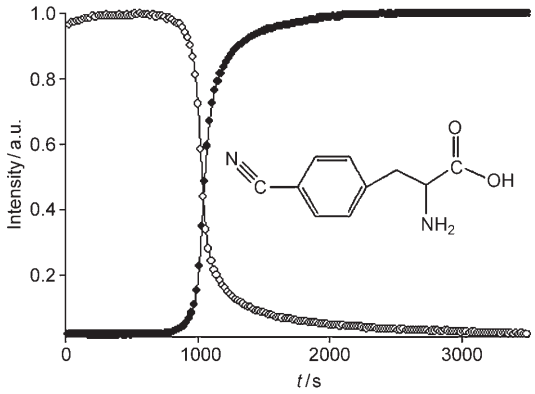

Figure 3. Comparison of the time course of thioflavin T fluorescence (●) at 480 nm and *p*-cyanoPhe fluorescence (○) at 296 nm for hIAPP-Y37F$_{C\equiv N}$ at pH 7.4, 25 °C. The data were normalized so that the total signal change is displayed on a scale of 0.0 to 1.0. The molecular representation of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

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 show 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; this rules out prefibrillar intermediates in which the C-terminal side chain is buried. The data presented here also show that the C-terminal side chain is partially sequestered from solvent. This offers an explanation for the apparent elevated pK_a for Tyr37 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 analogue described here should be a useful reagent for studies of fibrillization inhibitors since it avoids the problems associated with the use of extrinsic probes.[10]

Experimental Section

Wild-type hIAPP, hIAPP-Y37$F_{C\equiv N}$, and the Gly-$Phe_{C\equiv N}$-Ala-Ala tetrapeptide were synthesized as described.[11] The disulfide bond in IAPP was formed by using DMSO-based oxidation.[11] Fmoc-4-cyanoPhe was obtained from NovaBiochem. Peptides were purified by reverse phase HPLC and the identities confirmed by MALDI mass spectroscopy.

Thioflavin T fluorescence experiments were performed as described.[11] *p*-CyanoPhe was excited at 240 nm and fluorescence was detected at 296 nm with slit widths of 10 nm by using an Applied Photon Technologies fluorimeter. Stock solutions of hIAPP in HFIP (1.58 mM) were prepared as described.[12] Fibrillization reactions were initiated by diluting the stock 50-fold in buffered (20 mM Tris-HCl, pH 7.4) aqueous solution. Final conditions were hIAPP (32 μM), Tris-HCl (20 mM, pH 7.4), HFIP (2%), and thioflavin T (32 μM), when present.

TEM was performed at the University Microscopy Imaging Center (State University of New York, Stony Brook). Samples (4 μL) from the wild-type and hIAPP-Y37$F_{C\equiv N}$ 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 by using an Aviv 62A DS CD spectrophotometer. For far-UV CD wavelength scans, an aliquot from the peptide stock was diluted into Tris-HCl buffer (20 mM, pH 7.4). The final peptide concentration was 0.1 mg mL^{-1}. The spectrum is the average of three experiments in a 0.1 cm quartz cuvette, which were recorded over a range of 190–250 nm, at 1 nm intervals with an averaging time of 3 s per scan. A background spectrum was subtracted from the collected data.

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[1] a) G. G. Glenner, *N. Engl. J. Med.* **1980**, *302*, 1283–1292; b) J. D. Sipe, *Crit. Rev. Clin. Lab. Sci.* **1994**, *31*, 325–354; c) D. J. Selkoe, *Nat. Cell Biol.* **2004**, *6*, 1054–1061; d) F. Chiti, C. M. Dobson, *Annu. Rev. Biochem.* **2006**, *75*, 333–366.
[2] C. M. Dobson, *Trends Biochem. Sci.* **1999**, *24*, 329–332.
[3] a) C. Nilsberth, A. Westlind-Danielsson, C. B. Eckman, M. M. Condron, K. Axelman, C. Forsell, C. Stenh, J. Luthman, D. B. Teplow, S. G. Younkin, J. Naslund, L. Lannefelt, *Nat. Neurosci.* **2001**, *4*, 887–893; b) M. D. Kirkitadze, G. Bitan, D. B. Teplow, *J. Neurosci. Res.* **2002**, *69*, 567–577; c) C. Caughey, P. T. Lansbury, *Annu. Rev. Neurosci.* **2003**, *26*, 267–298.
[4] H. LeVine, 3rd, *Methods Enzymol.* **1999**, *309*, 274–284.
[5] T. Tomiyama, H. Kaneko, K. Kataoka, S. Asano, N. Endo, *Biochem. J.* **1997**, *322*, 859–865.
[6] a) M. J. Tucker, R. Oyola, F. Gai, *Biopolymers* **2006**, *83*, 571–576; b) Z. Getahun, C. Y. Huang, T. Wang, B. De Leon, W. F. DeGrado, F. Gai, *J. Am. Chem. Soc.* **2003**, *125*, 405–411.
[7] a) P. Westermark, C. Wernstedt, E. Wilander, D. W. Hayden, T. D. O'Brien, K. H. Johnson, *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 3881–3885; b) G. Cooper, A. C. Willis, A. Clark, R. C. Turner, R. B. Sim, K. B. M. Reid, *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 8628–8632; c) A. Lorenzo, B. Razzaboni, G. C. Weir, B. A. Yanker, *Nature* **1994**, *368*, 756–760; d) S. E. Kahn, S. Andrikopoulos, C. B. Verchere, *Diabetes* **1999**, *48*, 241–246.
[8] S. B. Padrick, A. D. Miranker, *J. Mol. Biol.* **2001**, *308*, 783–794.
[9] K. C. Schultz, L. Supekova, Y. H. Ryu, J. M. Xie, R. Perera, P. G. Schultz, *J. Am. Chem. Soc.* **2006**, *128*, 13984–13985.
[10] J. J. Meier, R. Kayed, C.-Y. Lin, T. Gurlo, L. Haataja, S. Jayasinghe, R. Langen, C. G. Glabe, P. C. Butler, *Am. J. Physiol. Endocrinol. Metab.* **2006**, *291*, E1317–E1324.
[11] A. Abedini, D. P. Raleigh, *Org. Lett.* **2005**, *7*, 693–696.
[12] A. Abedini, D. P. Raleigh, *Biochemistry* **2005**, *44*, 16284–16291.

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