

Preparation of synthetic human islet amyloid polypeptide (IAPP) in a stable conformation to enable study of conversion to amyloid-like fibrils

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Abstract Human synthetic islet amyloid polypeptide (hIAPP) is rapidly converted to β -sheet conformation and fibrils in aqueous media. Optimal solubility conditions for hIAPP were determined by circular dichroism spectroscopy and transmission electron microscopy. hIAPP in trifluoroethanol or hexafluoro-2-isopropanol (HFIP) diluted in water or phosphate buffer (PB) exhibited random structure which was converted to β -sheet and fibrils with time. hIAPP, solubilised in HFIP, filtered and lyophilised remained in stable random structure for up to 7 days in water; in PB, insoluble aggregates precipitated from which protofilaments and fibrils formed with time. This suggests that amorphous aggregates of hIAPP could initiate islet amyloidosis *in vivo*.

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Key words: Islet amyloid polypeptide; Fibril; β -Sheet; Amyloid

1. Introduction

Islet amyloid polypeptide (IAPP), amylin, is a 37 amino acid peptide which forms amyloid fibrils in pancreatic islets in type 2 diabetes [1,2]. The mechanisms promoting conversion of soluble monomeric IAPP into β conformation and fibril formation are largely unclear. Rodent IAPP, which differs from the human sequence in only six residues, is not amyloidogenic; this has been ascribed to the substitution of proline residues in the region IAPP^{20–29} [3–5]. *In vitro* investigations into the secondary structure of human IAPP and identification of the structural changes that accompany amyloid fibril formation have been hampered by the rapid conversion of the peptide into fibrils in aqueous media [6,7]. Reports of secondary structure of human IAPP have been inconsistent and have utilised material from different sources and prepared under varied conditions; overall these studies suggest that human synthetic IAPP, when prepared in aqueous

medium, assumes a largely β conformation and oligomerises whereas the more soluble rat IAPP is in random conformation [6–9]. However, when human IAPP was prepared in hexafluoroisopropanol (HFIP), a random conformation was observed which was rapidly converted to β conformation by ‘seeding’ with preformed fibrils [10,11].

Some of the difficulties encountered in attempting to reproduce the many and varied proposed physiological and cytotoxic effects of IAPP with synthetic peptide may have resulted from the difficulty in maintaining human IAPP in a stable monomeric conformation. To establish a reproducible method of preparation of human IAPP so that the peptide is in a stable molecular structure and can be used in physiological experiments, the molecular conformation and stability of different preparations of human IAPP have been studied by circular dichroism (CD) spectroscopy and fibril formation by electron microscopy and thioflavine T fluoroscopy.

2. Materials and methods

2.1. Secondary structure predictions of human and rat IAPP

Different algorithms were used for prediction of secondary structure of human IAPP^{1–37} and rat IAPP^{1–37}. These methods included double prediction [12], GOR4 [13], HNNC, SIMPA96 [14], SOPM [15] and Sec Cons.

2.2. Preparation of peptide solutions for examination of molecular structure

Human IAPP lyophilised from trifluoroacetic acid was obtained from Bachem (Torrance, CA, USA) or Peninsula Laboratories (St Helens, UK). Rat IAPP (Bachem) and the Alzheimer's peptide A β ^{1–42} (Bachem) were also examined as controls. Peptides were prepared in fluorinated alcohols (1 mg/ml) and molecular structures compared in diluted alcohols, in water or phosphate buffer (PB). Commercial samples of peptides were reconstituted either (i) in 60% 2,2,2-trifluoroethanol (TFE) (Sigma, Poole, UK) in water; or (ii) in 100% 1,1,1,3,3,3-hexafluoro-2-isopropanol (HFIP) (Sigma). Aliquots were either examined directly in diluted samples or freeze dried and reconstituted in TFE, HFIP, dH₂O or in 10 mM PB, pH 7.0. To further purify the peptide, some samples dissolved in HFIP were filtered (0.2 μ m) before lyophilisation.

2.3. CD spectroscopy

Aliquots of peptide solutions (200 μ l) were examined over 190–250 nm wavelength, in quartz cuvettes with a 1 mm path length in a Jasco J720 spectropolarimeter. The photomultiplier voltage was monitored to ensure that the samples were transparent. Baseline spectra (solvent only) were subtracted from collected data.

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2.4. Electron microscopy

Peptide preparations were supported on carbon- and formvar-coated grids and negatively stained with 2% uranyl acetate. Grids were examined with a Joel 1010 microscope, accelerating voltage 80 kV.

2.5. Thioflavine T spectroscopy

Samples of peptide in buffer (800 μ l) were mixed with 10 μ l thioflavine T (Th T) (1 M, Sigma, 20% dye content) and aliquots of the mixture examined over a 10-min period at an excitation wavelength of 350 or 450 nm and emission spectra collected at 400–500 nm in a spectrofluorometer (Perkin Elmer). Peaks of emission for unbound Th T were observed at 438 nm and at 482 nm for thioflavine bound to fibrils. Background readings from control solutions were subtracted. To determine conditions for optimal Th T binding to IAPP fibrils, fibril mixtures were examined at different pHs in 10 mM citrate buffer, pH 4, 10 mM PB, pH 7.4, 10 mM glycine–NaOH at pH 9 and 10.6.

3. Results

3.1. Secondary structure predictions

The different methods of structure prediction produced some common features (Fig. 1). The double prediction method (Fig. 1a, 1) indicated that human IAPP had a random sequence at the N-terminus followed by a helical segment; the short extended β -strand was predicted to extend to His¹⁸ and was separated from the longer β -strand in the region IAPP^{26–29} by a random conformation. These two β -strand regions were a feature of all but one of the predictions. A β turn was proposed by two systems in the region of IAPP²⁰ (Fig. 1a, 1,6). Rat IAPP had a similar helical segment towards the N-terminus in all predictions but the proline residues at

positions 24, 27 and 29 appear to disrupt the propensity for β -strand in this region (Fig. 1b).

3.2. Structural features of hIAPP solubilised in 60% TFE compared with rIAPP and A β

To improve the solubility and stability of hIAPP, the commercially supplied peptide (Bachem) was reconstituted in 60% TFE and freeze dried. TFE at high concentrations can promote helical structure and reduce the tendency for conversion to β -sheet and formation of insoluble fibrils [16,17]. The maximal proportion of TFE for hIAPP solubility was 60% and IAPP in this solvent was compared with IAPP in water. Dissolution of aliquots at 26 μ M in either 60% TFE or in dH₂O resulted in a spectrum with a minimum at 221 nm and a shoulder at 207 nm (Fig. 2). These features suggest a mixture of α -helical and β conformation. The samples were examined over a 24-h period (incubation at 20°C) when there was a progressive increase in the maximum at 196 nm and a further increase in the minimum which became closer to 218 nm (Fig. 2a). When prepared in 60% TFE human IAPP precipitated from solution over a 1-week period (Fig. 2b). Human IAPP (26 μ M) from Peninsula Laboratories prepared by the same method as above exhibited a similar spectrum but with low amplitude suggesting that much lower concentrations of peptide were available for analysis compared to those seen with peptide obtained from Bachem. For comparison, rat IAPP^{1–37} and A β ^{1–42} were reconstituted and solubilised in 60% TFE or dH₂O as above. Rat IAPP dissolved in water showed a largely random structure but the minimum was at 201 nm suggesting a partly helical component (Fig. 2c) where-

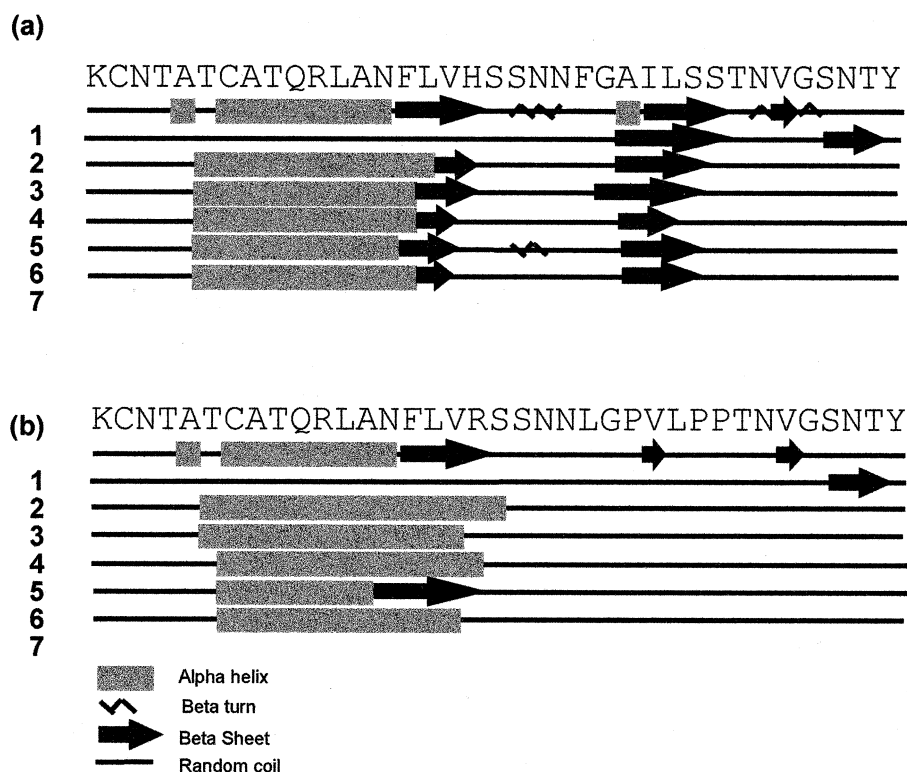


Fig. 1. Representation of secondary structure predictions for (a) human IAPP and (b) rat IAPP. Algorithms used: 1, double prediction; 2, GOR4; 3, HNNC; 4, Predator; 5, SIMPA96; 6, SOPM; 7, Sec Cons. Amino acid sequence of human IAPP (a) and rat IAPP (b) is indicated by single letter code.

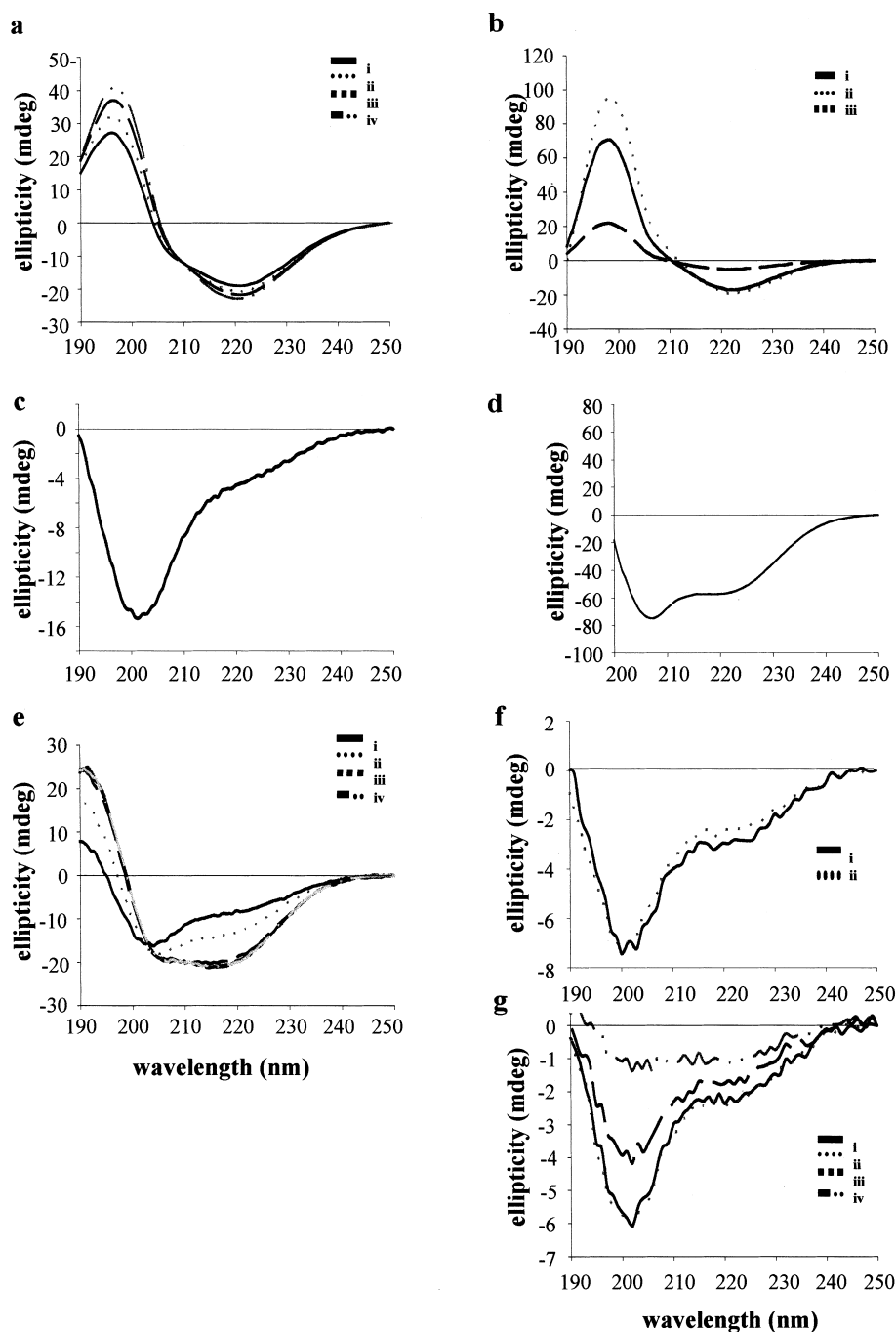


Fig. 2. CD spectra of human or rat IAPP prepared in water (a,c) or TFE (b,d) or solubilised in HFIP and reconstituted in 1% HFIP, water or PB (e–g). a: 26 μ M hIAPP (Bachem) in dH₂O at (i) time zero, solid line, (ii) 2 h, dotted line, (iii) 8 h, dashed line and (iv) 24 h showing a mixture of α -helix and β conformation but an increase in the β conformation with time. b: 26 μ M hIAPP reconstituted in 60% TFE measured over 1 week: (i) time zero, solid line, (ii) 6 h, (iii) 7 days showing that the peptide comes out of solution progressively. c: Spectrum of rat IAPP dissolved in dH₂O after 7 days incubation showing largely random conformation. d: Spectrum of hIAPP prepared and dissolved in 10% HFIP after 7 days incubation exhibiting a largely helical conformation. e: 12 μ M hIAPP in 1% HFIP in PB (i) time zero, solid line, (ii) 10 min, dotted line, (iii) 30 min, dashed line, (iv) 3 h, dash-dotted line; this was a less stable preparation and was gradually converted from α to β with time. f: 12 μ M hIAPP freeze dried from filtered 100% HFIP and reconstituted in water: (i) time zero and (ii) after 7 days, dotted line, showing that the peptide is in a stable random conformation. g: 12 μ M hIAPP freeze dried from filtered 100% HFIP and reconstituted in PB: (i) time zero, solid line, (ii) 1.5 h, dotted line, (iii) 3 h, dashed line, (iv) 24 h, dash-dotted line; the peptide gradually precipitated from the solution without any evidence of conversion to a β conformation.

as a classical helical spectrum was observed in 60% TFE: these conformations did not change after 7 days incubation at 20°C. Fibrils were visible by transmission electron microscopy (TEM) at all time points in mixtures of hIAPP in 60%

TFE or dH₂O, or in aliquots of A β ^{1–42} after 24 h incubation: these fibrils were straight and unbranching with a diameter of 5–10 nm. However, no fibrils or precipitate were present in the rat IAPP solutions.

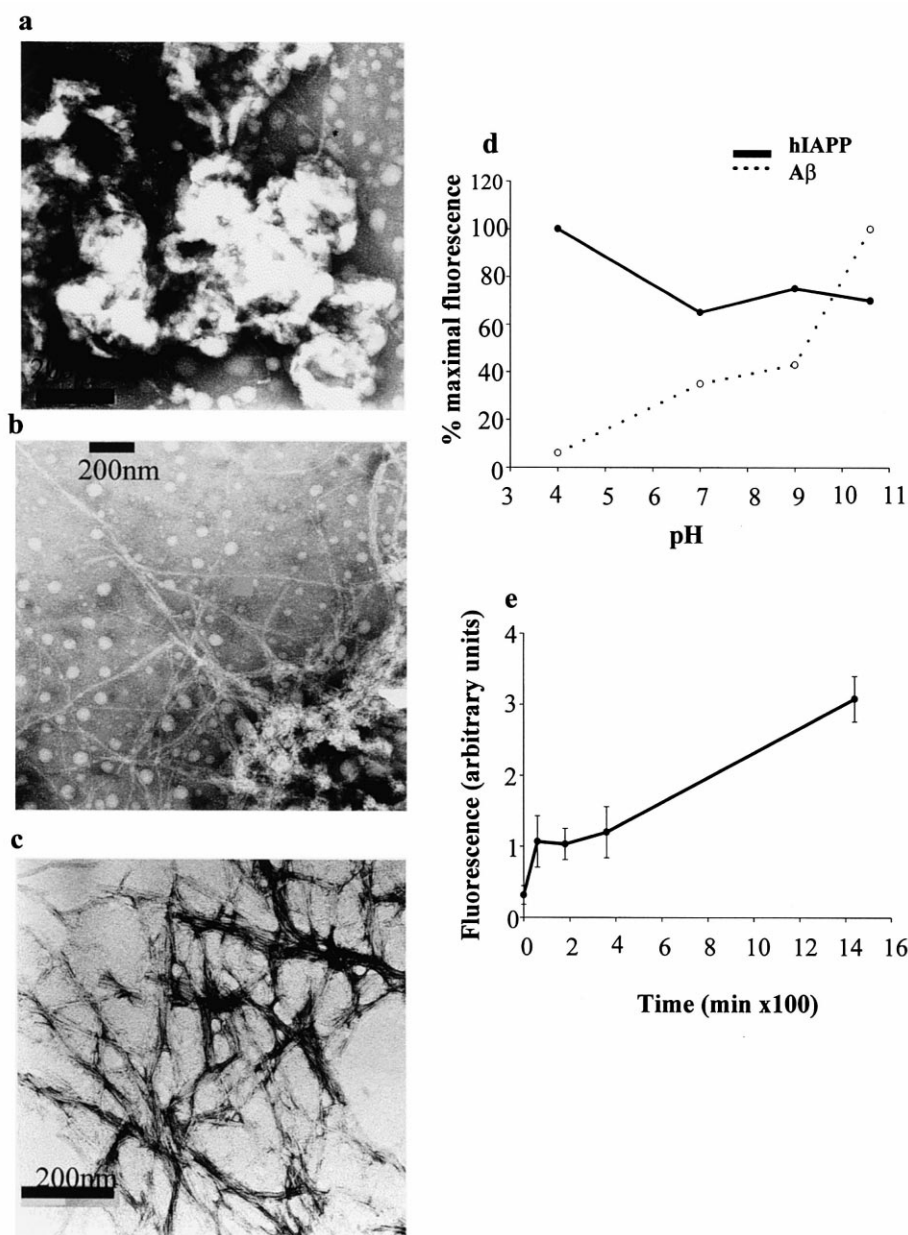


Fig. 3. Development of fibrils from aggregates of hIAPP precipitated in PB. a–c: Electron micrographs of negatively stained preparations of hIAPP lyophilised from HFIP and reconstituted in PB (a), after 60 min when small protofilaments were forming from the aggregates (b), after 180 min when there was a mixture of amorphous and protofilaments (c) and at 24 h when the aggregates had been completely replaced by fibrils (d). pH dependence of Th T binding to fibrils of hIAPP (upper panel) and Aβ^{1–42} (6 μM) and (lower panel) time course of fibril formation of hIAPP lyophilised from HFIP and reconstituted in PB, same preparations as in a–c, mean ± S.E.M., *n* = 3.

3.3. Effect of solubilisation of hIAPP with HFIP

Since hIAPP was soluble in 100% HFIP this was potentially a more effective solvent than TFE to maintain helical conformation and prevent fibril formation [18,19]. hIAPP (65 μM) solubilised in 100% HFIP and diluted to 10% HFIP in dH₂O (6.5 μM hIAPP) exhibited a largely helical spectrum. The molecular structure of hIAPP in 10% HFIP was stable, in a largely helical conformation, after a 1-week incubation at 20°C (Fig. 2d) and no fibrils were detected with TEM. hIAPP in 1% HFIP in PB was less stable and conversion to a mixture of helix and β structure via a random conformation was apparent after 30 min incubation (Fig. 2e).

However, 1% HFIP is toxic to cultured cells so this method of IAPP preparation is not suitable for physiological exper-

imentation, therefore the effects of excluding HFIP from the final mixture were determined. A filtration step (0.2 μm filters) was introduced to remove potential 'seeds' for aggregation [11,20]. Following reconstitution of hIAPP (Bachem) in 100% HFIP and filtration, aliquots of hIAPP were freeze dried to remove the HFIP. These were then prepared in water (pH 7.3) or PB (pH 7.5) at 6.5 μM. In dH₂O, hIAPP exhibited a random spectrum with slight helical properties (Fig. 2f); this spectrum remained unchanged in characteristics or amplitude over the 7 days of examination and fibrils were not seen by TEM at any time point. Samples prepared in PB had a similar structure to those in dH₂O but the spectra were not stable with incubation: there was almost complete loss of signal after 24 h at 20°C (Fig. 2g) without evidence of a change to

β conformation. Samples prepared in PB contained amorphous protein aggregates visible by TEM at the initial time point but there was no evidence of fibrils (Fig. 3). After 1 h incubation the aggregates increased in density but occasional fine protofilaments were visible (Fig. 3a,b) which increased in number over the following hours and, at 24 h, the preparation contained predominantly fibrils with the typical amyloid characteristics (Fig. 3c). Thus, IAPP can be in a stable unfolded state in water at neutral pH in the absence of ‘seeds’. However, addition of phosphate salts promoted precipitation into amorphous aggregates and eventually fibril formation.

3.4. Examination of fibril formation with thioflavine T fluorescence

Further evidence of precipitation as amorphous aggregates came from samples prepared in PB and examined by Th T fluorescence to quantify fibril formation. To optimise the conditions for binding of Th T to IAPP fibrils, the pH dependence of binding was determined with a standard mixture of IAPP fibrils and compared with a fibril-containing preparation of A β ^{1–42} (1 mg/ml). Samples of A β ^{1–42} and hIAPP (0.26, 1.5, 3 and 6 μ M) were examined in the absence of Th T to determine the contribution of light scattering to the signal. This was proportional to the concentration of peptide but similar at all pHs examined. Whereas the binding of Th T to A β ^{1–42} increased with a change in pH from 4 to 11 there was no pH dependence for Th T binding to hIAPP (Fig. 3d). Subsequent experiments were made at pH 9.0 to be consistent with other reports [22–24]. Samples of hIAPP prepared in PB from the HFIP-filtered and solubilised stock (as examined above with CD) (50 μ g/ml) exhibited no significant fluorescence at time zero indicating the absence of fibrils. However, the Th T binding gradually increased, reaching a maximum after 24 h incubation (Fig. 3e). This corresponds to the electron microscopic observations of no fibrils at time zero followed by fibril growth from amorphous aggregates over this time period.

4. Discussion

These data suggest that synthetic human IAPP can exist in a stable, largely random conformation in aqueous medium when all potential ‘seeding’ material has been removed. In phosphate buffer, hIAPP precipitated as amorphous aggregates without observable fibril formation. Protofilaments developed from these aggregates to form typical amyloid fibrils over periods of time.

Previously reported secondary structure predictions for hIAPP^{1–37} have indicated a short region of propensity for β -strand in the region IAPP^{25–29} [6,10]. The majority of predictions for rat IAPP show no evidence for substantial regions of β -strand either in the amino acid substituted region, IAPP^{20–29}, or in the region homologous with the human peptide, IAPP^{13–16}. However, such predictions are suggestions only and folding studies and crystal structure analyses are required to confirm molecular conformation.

TFE is known to promote α -helical tendencies by affecting intra-molecular hydrogen bonding and destabilising tertiary contacts [17,19]. It was therefore examined as a solvent to create a soluble state of hIAPP which did not form fibrils immediately. Whereas rat IAPP and A β peptide were converted from a random conformation to classical α -helices

when solubilised in 60% TFE, the β structure of hIAPP was unchanged and fibrils were present at all time points. This is consistent with the data of Hubbard et al. [7]; however, there was no evidence for a large, 98%, α -helical contribution as described previously [9]. Differences between reported data and the present results may reflect the relative proportions of monomeric, oligomeric and fibrillar peptide that are present in the CD cuvette under conditions of fibril formation; the different proportions of these oligomers in a mixture could account for the many different structures that have been reported for hIAPP prepared in water or in TFE.

hIAPP was more soluble in HFIP than in TFE and in 100% HFIP produced an α -helical signal which was stable over many days. Reduction of the proportion of HFIP to 1% in PB destabilised the molecule which was initially converted to a more random structure as has been described previously [11,21] and finally to a β structure with the appearance of fibrils after 24 h incubation. Filtration of the peptide in HFIP before freeze drying removed material >0.2 μ m which could act as ‘seeds’ for initiation of fibril formation [20,21]. Amino acid analyses demonstrated that less than 10% of the original concentration of peptide was removed by filtration; this indicated that the absence of fibrils when reconstituted in water was not as a result of a reduction in concentration. Thus synthetic hIAPP, like rat IAPP and the homologous peptide, calcitonin gene-related peptide [7], can exist in a stable random structure in the absence of material which has the potential to seed the fibril-forming reaction. From this conformation, a degree of unfolding and refolding would be required during the process of amyloid fibril formation. Unfolding of hIAPP has been described under strong denaturing conditions (6 M GdnHCl) and at high temperatures (60°C) [21]; under moderate reducing conditions, a population of IAPP in partially folded states has been described which rapidly forms fibrils. However, the process by which hIAPP is converted to fibrils from stable monomeric form remains unclear.

When hIAPP (lyophilised and filtered but in the absence of the stabilising 1% HFIP) was reconstituted in PB, the peptide initially exhibited a random conformation as in water. However, over a 24-h period, the peptide precipitated out of solution without exhibiting, at any time, a β -sheet conformation or any other intermediate form as has been suggested for other amyloidogenic peptides [25]. The amorphous aggregate resulting from this precipitation was visible by TEM. The very fine (~6 nm diameter) protofilaments that formed amongst this precipitate could represent the very earliest stages of fibril formation. This process, which was confirmed by Th T fluorescence, increased with time so that, by 24 h, classical amyloid fibrils had replaced the aggregate.

These data suggest that hIAPP can exist in a stable random form in aqueous media in the absence of ‘seeds’ but will precipitate, as an unstructured aggregate which in turn creates a favourable microenvironment for conversion of hIAPP to β -sheet and protofilament formation. This sequence of events could be analogous to changes occurring in the deposition of islet amyloid and in other amyloidoses in vivo: amorphous accumulations of peptide (possibly not in β conformation) have been described as an early step in the process of fibrillogenesis in pancreatic islets [26] and in Alzheimer’s disease [27]. These accumulations could form the matrix for protofilament formation and assembly of fibrils in vivo.

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