Supporting Information for

Ester to Amide Switch Peptides Provide A Simple Method for Preparing Monomeric Islet Amyloid Polypeptide Under Physiologically Relevant Conditions and Facilitate Investigations of Amyloid Formation.

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EXPERIMENTAL PROCEDURES

Peptide Synthesis. Human IAPP, rat IAPP, the Ser-20-IAPP switch peptide, and a small switch peptide, VHSSNN, corresponding to residues 17 to 22 of human IAPP with the Ser-Ser amide linkage replaced by an ester were synthesized on a 0.25 mmol scale using an Applied Biosystems 433A peptide synthesizer, via 9-fluornylmethoxycarbonyl (Fmoc) chemistry. Solvents used were A.C.S. grade. Fmoc protected pseudoproline (oxazolidine) dipeptide derivatives and Boc protected isoacyl dipeptide derivatives were purchased from Novabiochem. All other reagents were purchased from Advanced Chemtech, PE Biosystems, Sigma, and Fisher Scientific. A 5-(4'-fmoc-aminomethyl-3', 5-dimethoxyphenol) valeric acid (PAL-PEG) resin was used to form an amidated C-terminus. Standard Fmoc reaction cycles were used expect for the coupling of Boc-Ser(Fmoc-Ser(tBu))-OH dipeptide derivative at residues 19 and 20. The Boc-Ser(Fmoc-Ser(tBu))-OH isoacyl dipeptide derivative was double coupled under non-basic conditions using N-hydroxybenzotriazole (HOBT) / N, N'-Diisopropyl-carbodiimide (DIPCDI) (1:1.1) in dichloromethane (DCM) / dimethylformamide (DMF) (3:1). The first residue

attached to the resin, pseudoproline dipeptide derivatives, all β-branched residues, and all residues directly following a β-branched residue were double coupled. Peptides were cleaved from the resin using standard trifluoroacetic acid (TFA) methods.

Peptide Purification and Oxidation. Crude peptides were partially dissolved in 20% acetic acid (v/v), frozen in liquid nitrogen and lyophilized. This procedure was repeated several times prior to purification to increase solubility. Disulfide bond formation was induced via oxidation by DMSO. The peptide was dissolved in 99% DMSO, 1% HCl and allowed to stand at room temperature for a minimum of 5 days. Disulfide bond formation was monitored by analytical HPLC. The oxidized peptide was lyophilized. The dry peptides were then re-dissolved in 30% acetic acid (v/v) and purified via reversed-phase HPLC, using a Vydac C18 preparative column (10 mm × 250mm). A two-buffer system was used: buffer A consists of 100% H_2O and 0.045% HCl (v/v) and buffer B includes 80% acetonitrile, 20% H_2O and 0.045% HCl (v/v). HCl was utilized as the ion pairing agent instead of TFA since TFA can influence the rate of aggregation of amyloidogenic peptides. Purity was checked by HPLC using a Vydac C18 reversed-phase analytic column (4.6mm × 250mm) before each experiment. This is important because IAPP can undergo spontaneous deamidation. Peptides were analyzed by mass spectrometry using a Bruker MALDI-TOF MS. Oxidized human IAPP; expected 3903.6, observed 3903.7. Oxidized rat IAPP; expected 3921.3, observed 3921.6. Oxidized Ser-20-IAPP switch peptide; expected 3903.6, observed 3903.4. Small switch peptide; expected 697.6, observed 699.0.

Thioflavin-T-Binding Kinetic Experiments. Thioflavin-T binding assays were used to measure the development of structurally ordered fibrils over time. All fluorescence experiments were performed on an Applied Phototechnology fluorescence spectrophotometer using an excitation wavelength of 450 nm and an emission wavelength of 485 nm. The excitation and emission slits were 6 nm. A 1.0 cm cuvette was used. Solutions were prepared by dissolving dried peptide into 20 mM pH 4.2 Glycine-HCl buffer and thioflavin-T solution immediately before the measurement. Samples were filtered using a GHP Acrodisc 13mm Syringe filter with a 0.45 µm GHP membrane. The conditions were 16 µM Ser-20-IAPP switch peptide, 25 µM Thioflavin-T, 25°C for all experiments. Sodium hydroxide (NaOH) solution was used to adjust the pH value from 4.2 to the final values. All solutions were stirred during these experiments in order to maintain homogeneity.

Circular Dichroism (CD). CD spectra were measured on an Applied Photophysics Chirascan circular dichroism spectrometer. For far-UV CD wavelength scans, the peptide solutions were prepared by dissolving the dried peptide into 20 mM Glycine-HCl buffer at pH 4.2. The final peptide concentrations for far-UV CD experiments were 16 µM. Spectra were recorded from 195 to 260 nm at 1 nm intervals in a quartz cuvette of 0.1 cm path length at 25°C. CD experiments used the same peptide solutions that were used for the thioflavin-T fluorescence experiments.

Transmission Electron Microscopy (TEM). TEM was performed at the Life Science Microscopy Center at the State University of New York at Stony Brook. TEM samples were prepared from the solutions used for the fluorescence measurements. 15 µL of the peptide solution was removed and placed on carbon-coated formvar 200 mesh copper grid for 1 min and then negatively stained with saturated uranyl acetate for 1 min.

Dynamic Light Scattering (DLS). Dynamic light scattering experiments were performed with a 90Plus particle size analyzer (Brookhaven Instruments Corporation) at 25°C. The particle size distribution was determined at an initial peptide concentration, (determined by absorbance) of 100 µM Ser-20-IAPP switch peptide in 20 mM pH 4.2 Glycine-HCl buffer. Samples were filtered using a GHP Acrodisc 13mm Syringe filter with a 0.45 µm GHP membrane before the measurement. Rat IAPP, which does not aggregate, was used as a control at the same concentration as the Ser-20-IAPP switch peptide. The measured intensity autocorrelation function was converted to an effective size distribution using the software supplied by the manufacturer.

Ultra filtration experiments. Ser-20-IAPP switch peptide in 20 mM pH 4.2 Glycine-HCl buffer was centrifuged using a Microcon centrifugal filter device with an Ultracel YM-10 membrane (molecular weight 10,000 cut off) for 30 minutes at 14,000g, 25°C. The UV absorbance was measured at 280nm before and after the

centrifugation using a DU 730 Life Science UV/Vis spectrophotometer (Beckman Coulter). Rat IAPP, which does not aggregate, was used as a control.

Gel Filtration. Gel Filtration was performed with an AKTA purifier 10 FPLC (GE Healthcare) at 4°C using a superdex 75 10/300 GL column. The flow rate was set to 0.5ml/min. The apparent molecular masses were determined at initial peptide concentrations of 100 µM Ser-20-IAPP switch peptide in 20 mM pH 4.2 Glycine-HCl buffer. 20 mM Glycine-HCl buffer (pH 4.2) with 0.15 M NaCl was used as the elution buffer. Rat IAPP, which does not aggregate, was used as control at the same concentration as the Ser-20-IAPP switch peptide. Elution profiles were monitored at both 220 nm and 280nm. The amount of peptide which elutes in the observed peak was determined by integrating the 280 nm trace. The 280 nm trace was used since the extinction coefficient of IAPP is known at this wavelength.

Analytical Ultracentrifugation (AUC). Analytical ultracentrifugation was performed with a Beckman Optima XL-A analytical ultracentrifuge at 25°C using rotor speeds of 38,000 rpm (24h) and 48,000 rpm (24h). Apparent molecular masses were determined at initial peptide concentrations of 30, 60, and 100 µM Ser-20-IAPP switch peptide in 20 mM Glycine-HCl buffer (pH 4.2). A six channel, 12 mm path length, charcoal-filled Epon cell with quartz windows were used. The absorbance was measured at 280 nm and five scans were averaged. The partial specific volume (0.7146 mL g^{-1}) and solution density (1.0025 g l^{-1}) were calculated from the software

program SEDNTERP. The HeteroAnalysis program from the Analytical Ultracentrifugation Facility at the University of Connecticut was used for data analysis.

HPLC Based Assays of the kinetics of the O to N acyl shift. We used an HPLC based assay to measure the rate for the conversion of the ester to the amide in the full length 37 residue switch peptide and in a small switch peptide corresponding to residues 17 to 22 of human IAPP, VHSSNN, with an ester linkage between the two serines. The small peptide allows us to determine if the rate of conversion is dominated by local sequence effects, or if potential interactions, involving residues within the full length 37 residue switch peptide influence the rate. To avoid confusion, the small switch peptide will be referred to as the hexapeptide fragment and the full length 37 residue peptide construct as the switch peptide. After purification and lyophilized, samples were dissolved in 20 mM pH 7.4 Tris-HCl buffer to induce the O to N acyl shift (100 μM). At the desired time points (0 s, 30 s, 1 min, 2 min, 3 min, 4 min, 5 min), the reaction was quenched by adding 20% acetic acid to lower the pH value to 2.0. Then part of this mixture (0.5 ml) was directly analyzed by HPLC using a Vydac C18 reversed-phase analytical column (4.6mm × 250mm) with detection at 220 and 280 nm, using a gradient of 20% to 80% buffer B in 60 minutes. Buffer A consists of 100% H_2O and 0.045% HCl (v/v) and buffer B includes 80% acetonitrile, 20% H_2O and 0.045% HCl (v/v). The ester and amide forms are well resolved under these conditions.

RESULTS

The Ser-20-IAPP switch peptide is monomeric at pH 4.2. Four independent methods were used to determine the association state and distribution of species present in the pH 4.2 sample of the Ser-20-IAPP switch peptide. Rat IAPP which is known to be monomeric was used as control.

Dynamic light scattering experiments were performed to determine the particle size distribution. The observed intensity autocorrelation functions (Figure S1) of the Ser-20-IAPP switch peptide and rat IAPP are identical. The observed particle size distribution for the Ser-20-IAPP switch peptide (Figure S2) is similar to that of rat IAPP with a major peak at the value expected for a monomer and a very narrow distribution. No larger size aggregates were detected.

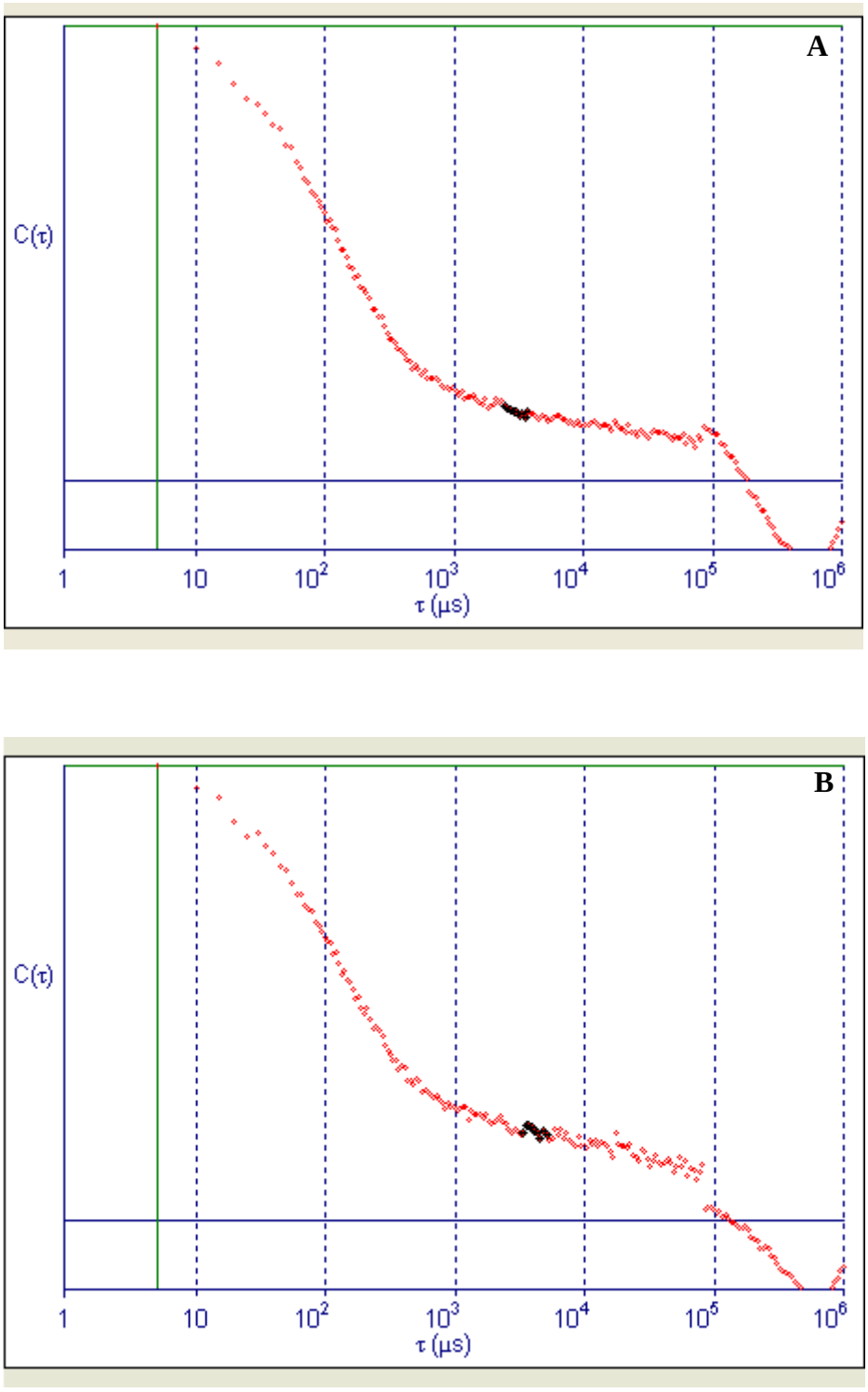

Figure S1: Intensity autocorrelation functions measured by DLS are displayed for (A) the Ser-20-IAPP switch peptide and (B) rat IAPP. Rat IAPP is known to be monomeric.

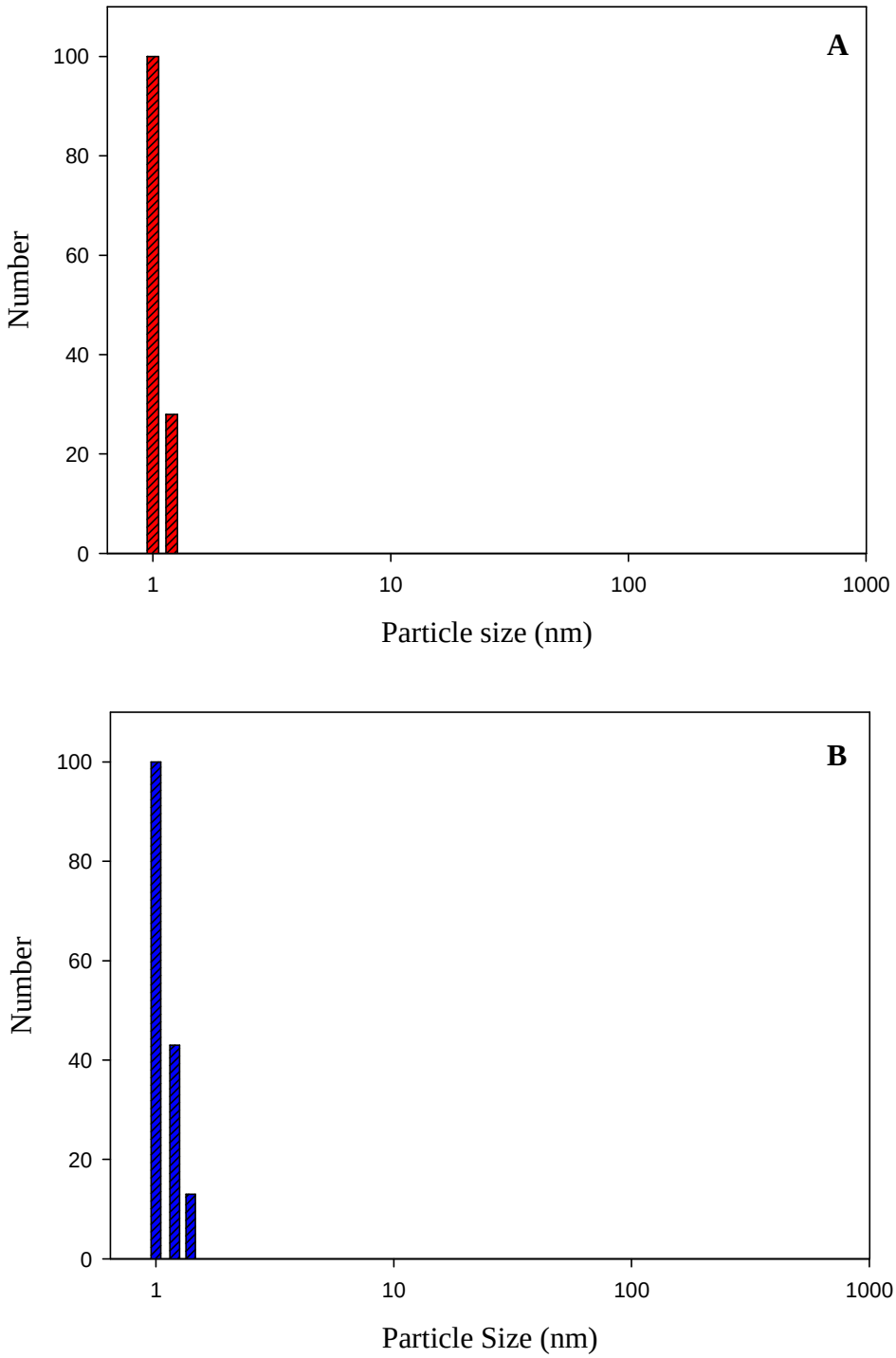

Figure S2: Dynamic light scattering demonstrates that the Ser-20-IAPP switch peptide is monomeric. DLS experiments were performed with a 90Plus particle size analyzer. Rat IAPP which is known not to aggregate has the same mean diameter as the Ser-20-IAPP switch peptide. Experiments were repeated five times. (A): Size distribution for the Ser-20-IAPP switch peptide; (B): Size distribution for the rat IAPP. Notice that no larger size particles were detected.

Gel filtration studies (Figure S3) indicate that the Ser-20-IAPP switch peptide is monomeric. Only a single peak is detected in the FPLC trace which has a retention time identical to rat IAPP which is known to be monomeric. Notice that no earlier eluting material is detected and no detectable material eluted in the void volume. The percentage of the total amount of the Ser-20-IAPP switch peptide which elutes in the observed peak was determined by integrating the area under the peak. Experiments were repeated three times and rat IAPP was used as a control. 96 ± 3% of the injected Ser-20-IAPP switch peptide elutes in the single observed peak.

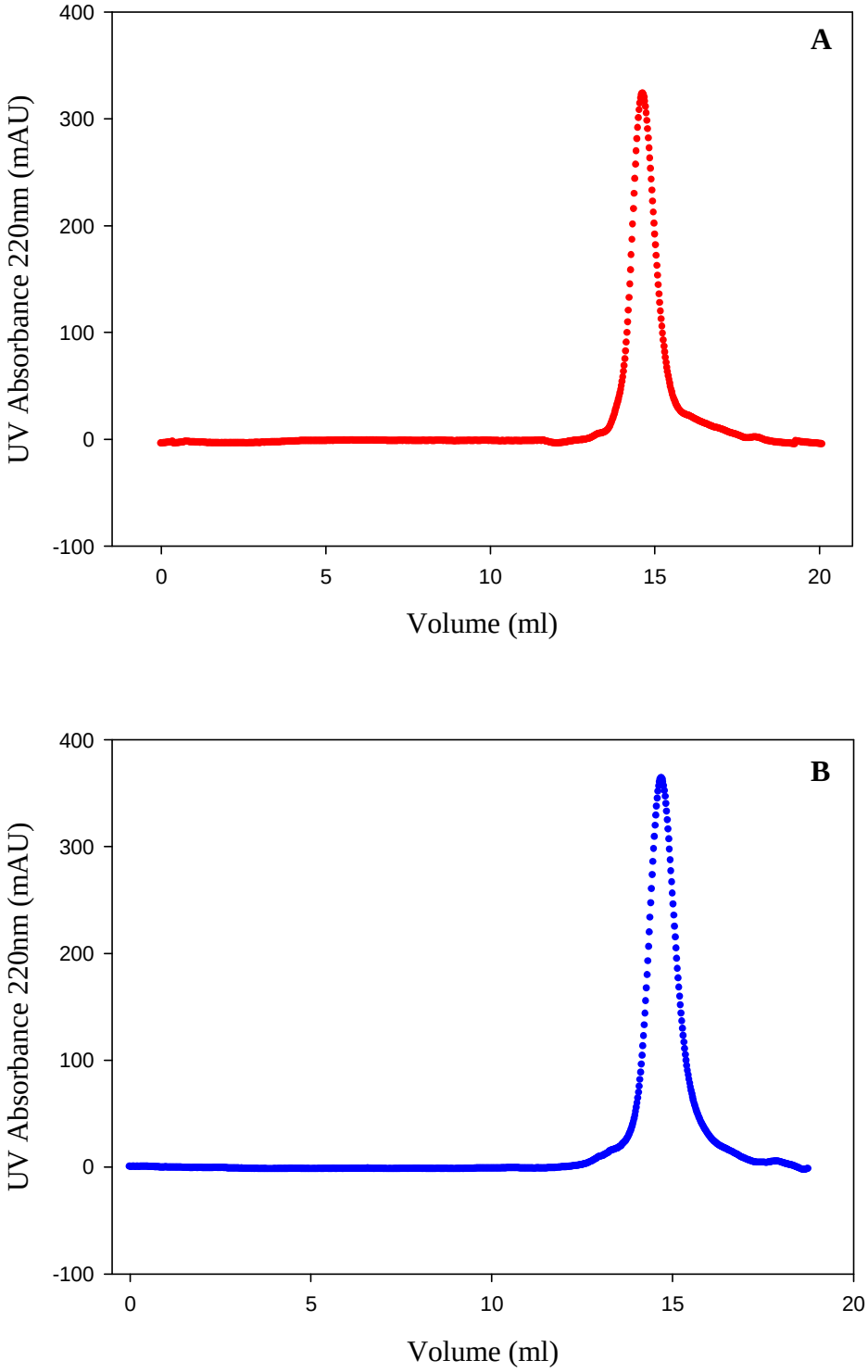

Figure S3: Gel filtration indicates that the Ser-20-IAPP switch peptide is monomeric. Gel filtration experiments were performed on a superdex 75 10/300 GL column. Rat IAPP which is known not to aggregate has the same elution time as the Ser-20-IAPP switch peptide. (A): Ser-20-IAPP switch peptide; (B): rat IAPP. Notice that no material elutes in the void volume of the column or prior to the observed peak.

Ultra filtration experiments were conducted using a microcon centrifugal filter device with a 10,000 molecular weight cut off membrane. The absorbance of the sample was measured at 280 nm prior to loading and after centrifugation for 30 minutes at 14,000 g. 93 ± 2% of the peptide passed through the membrane. Experiments were repeated three times. Rat IAPP was used as a control.

AUC experiments (Figure S4) confirm the results of the gel filtration, DLS and ultra filtration studies. The Ser-20-IAPP switch peptide is monomeric, based on single species fits to data collected at multiple concentrations at 38,000 rpm and 48,000 rpm.

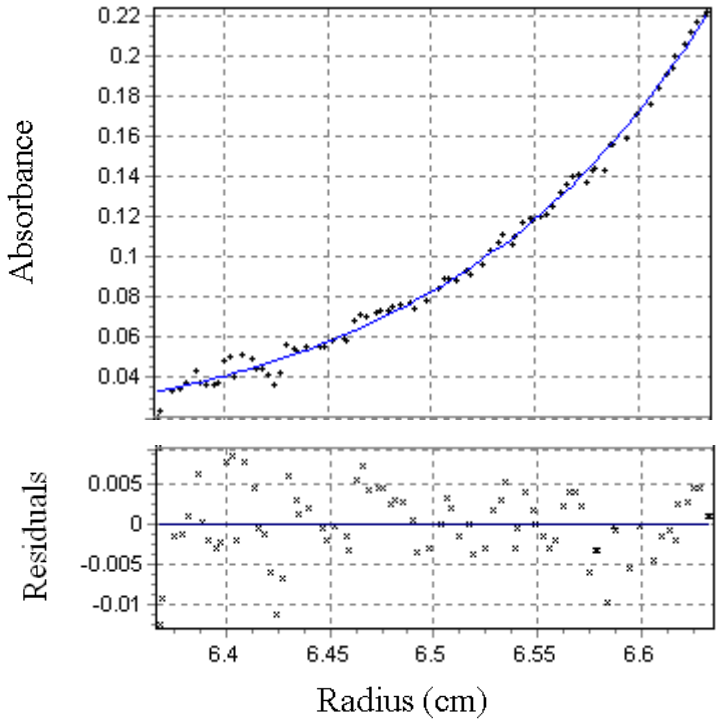

Figure S4: Equilibrium AUC of the Ser-20-IAPP switch peptide. The curve is well fit by a single species model with an apparent molecular weight of 4017, which is within ± 3% of the known monomer molecular weight of 3903. The sample was equilibrated at 48,000 rpm for 18 hours and was judged to be at equilibrium because subsequent scans collected at 20, 22 and 24 hours were super imposable with the 18 hour trace. A plot of the residuals is shown below the experimental trace.

The fact that equilibrium was reached is consistent with the hypothesis that the AUC experiment is monitoring the distribution of species in solution when the sample was loaded, and not simply material which transiently remains in solution after high molecular weight aggregates or fibrils are initially pelleted. Sedimentation equilibrium experiments reported by Hofrichter and coworkers on wildtype human IAPP at pH 4.9 have shown that the system fails to reach equilibrium because the amount of soluble IAPP is gradually depleted as high molecular weight aggregates are formed and continuously pelleted.[1] The fact that the sedimentation runs with the Ser-20-IAPP switch peptide reach equilibrium with a single, (monomeric), species present is consistent with the gel filtration, DLS, and ultra filtration studies.

The rate of the O to N acyl shift is rapid compared to the rate of amyloid formation. The data for the HPLC analysis of the Ser-20-IAPP full length switch peptide is shown in Figure S5 and the fit used to extract the kinetic parameters is shown in Figure S6.

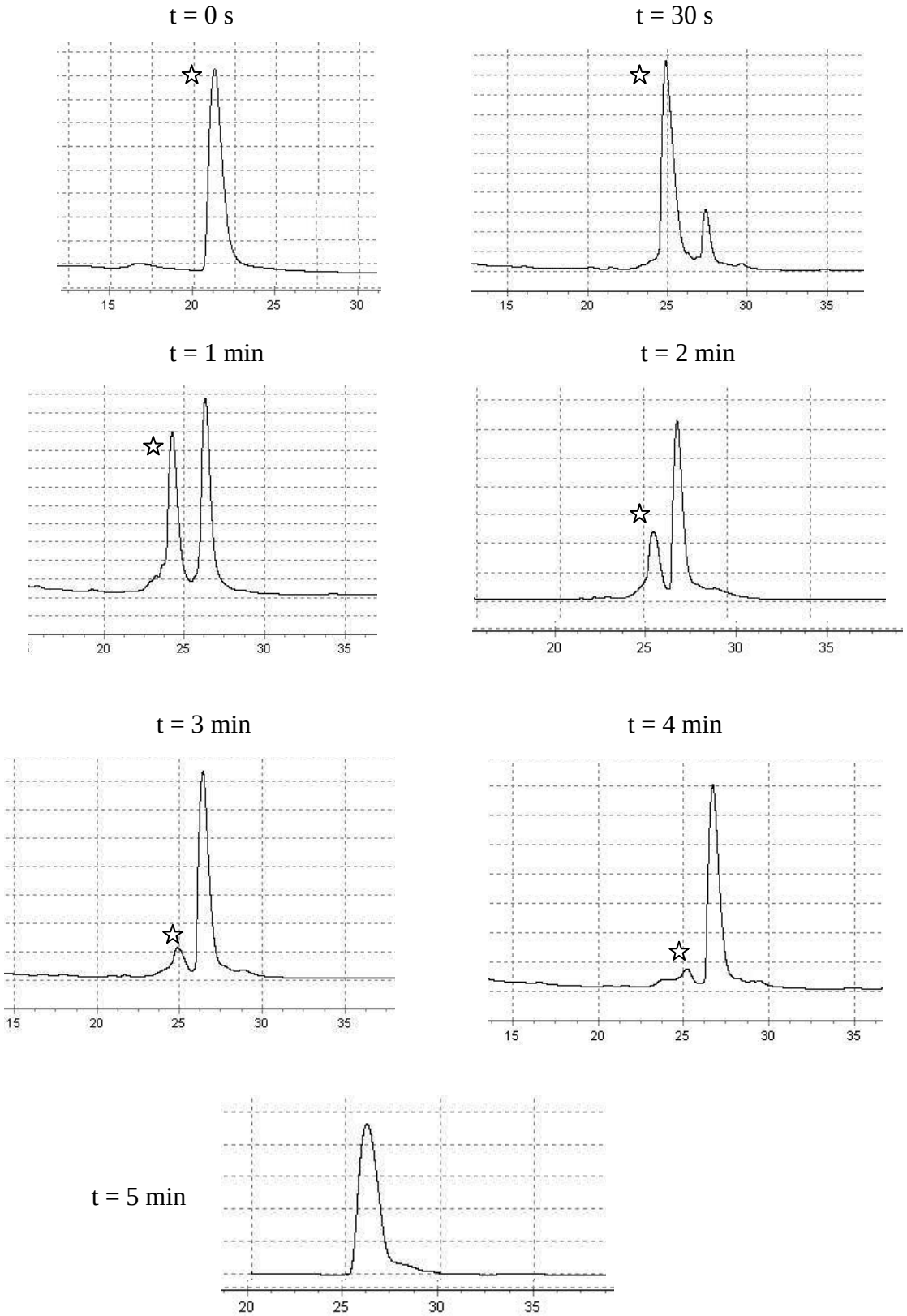

Figure S5: Kinetics of the O to N acyl shift of full length switch peptide monitored by HPLC. The peaks due to the ester form are indicated with a ☆ .

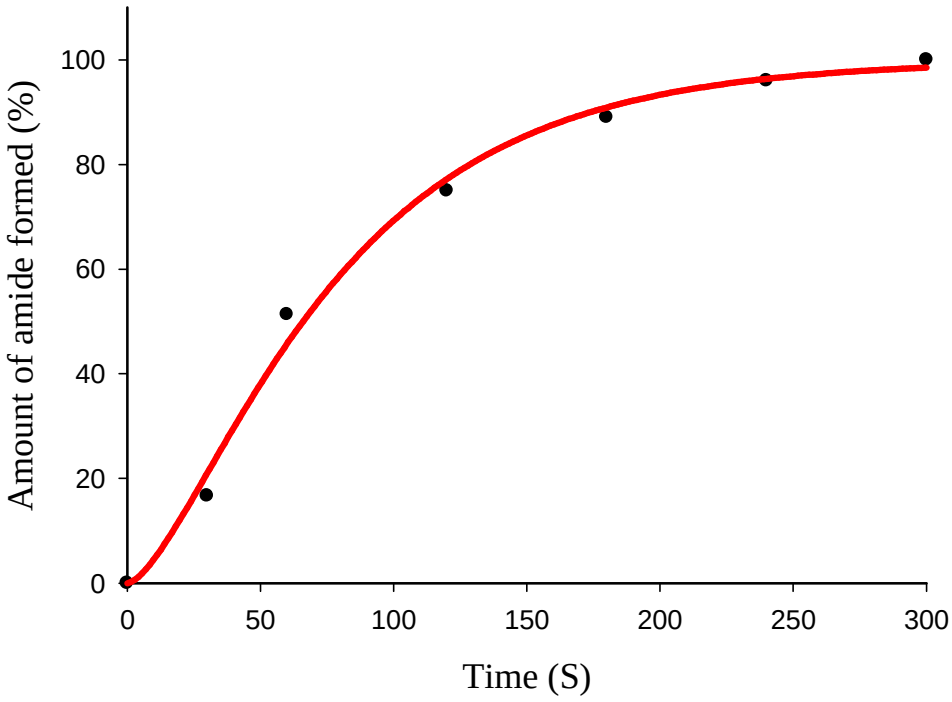

Figure S6: Time course of the conversion from the ester to amide form of the full length Ser-20-IAPP switch peptide following the pH jump. The curve is well fit by a single exponential (red) yielding a $t_{1/2}$ of 70 seconds. The data points are indicated as black circles.

The kinetics of conversion was also determined for the hexapeptide fragment using the same HPLC based approach. The fit to the data is shown in Figure S7. The observed rates for the conversion are essentially identical for the full length 37 residue switch peptide and the hexapeptide fragment. This indicates that any interactions involving residues outside of the hexapeptide region do not alter the kinetics. The important point is that the conversion is complete within 5 minutes while the lag phase for amyloid formation is on the order of 24 hours.

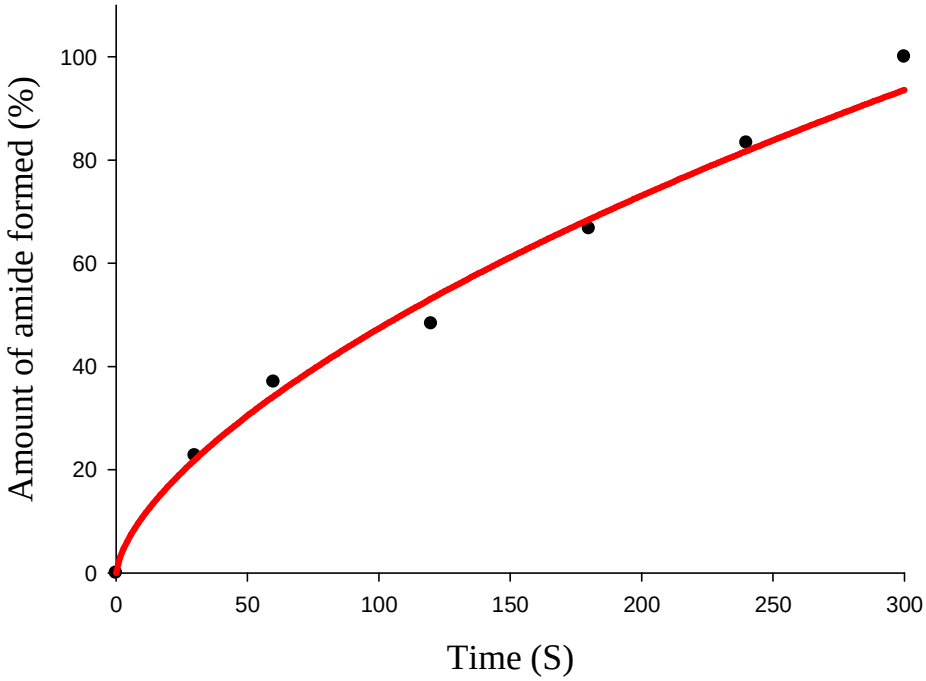

Figure S7: Kinetics of the O to N acyl shift for the hexapeptide fragment monitored by HPLC. Time course of the conversion from the ester to amide form following the pH jump. The curve is well fit by a single exponential (red) yielding a $t_{1/2}$ of 110 seconds. The data points are indicated as black circles.

Wild type human IAPP forms amyloid very rapidly when dissolved directly in buffer.

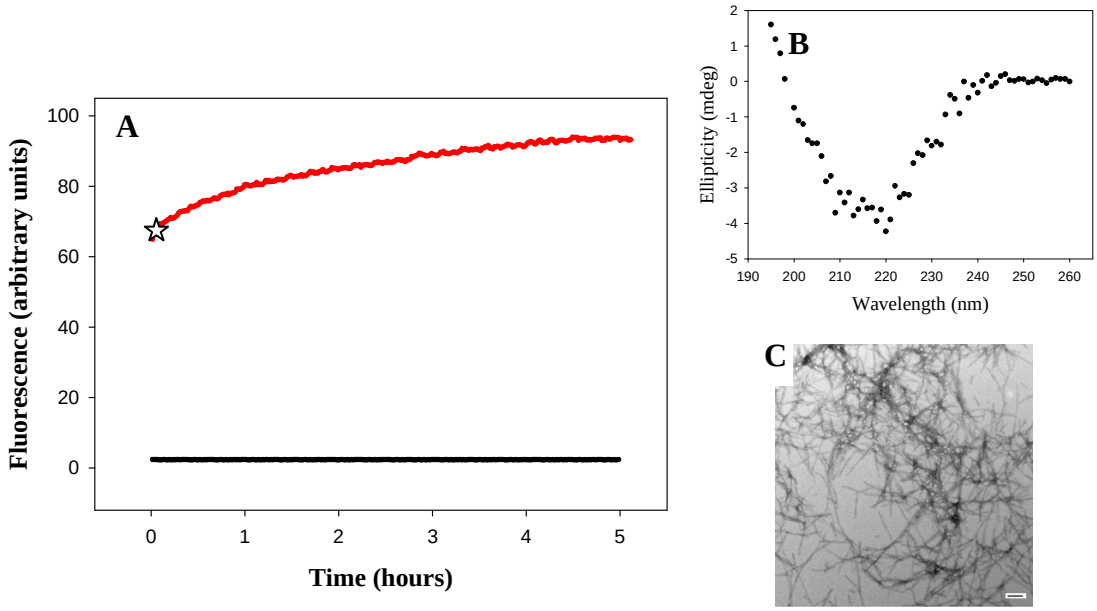

Figure S8: A dried sample of normal wild type human IAPP aggregates rapidly after dissolving in pH 7.4 buffer at a final peptide concentration of 16 µM. (A) Thioflavin-T fluorescence vs time. Red curve: wild type human IAPP dissolved in pH 7.4 buffer. Black curve: the Ser-20-IAPP switch peptide dissolved in pH 4.2 buffer. (B) The CD spectrum of wild type human IAPP shows β-sheet structure. (C) TEM image recorded of an aliquot which was removed immediately after the peptide was dissolved in buffer. The CD spectrum and TEM image are recorded at the time point indicated by the ☆. The scale bar in the TEM image represents 100 nanometers.

References:

(1) Vaiana, S. M., Ghirlando, R., Yau, W. M., Eaton, W. A., and Hofrichter, J. *Biophys J.* **2008**, *94*, L45-L47.