Nucleation and Growth of Insulin Fibrils in Bulk Solution and at Hydrophobic Polystyrene Surfaces

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ABSTRACT A technique was developed for studying the nucleation and growth of fibrillar protein aggregates. Fourier transform infrared and attenuated total reflection spectroscopy were used to measure changes in the intermolecular β-sheet content of bovine pancreatic insulin in bulk solution and on model polystyrene (PS) surfaces at pH 1. The kinetics of β-sheet formation were shown to evolve in two stages. Combined Fourier transform infrared, dynamic light scattering, atomic force microscopy, and thioflavin-T fluorescence measurements confirmed that the first stage in the kinetics was related to the formation of nonfibrillar aggregates that have a radius of 13 ± 1 nm. The second stage was found to be associated with the growth of insulin fibrils. The β-sheet kinetics in this second stage were used to determine the nucleation and growth rates of fibrils over a range of temperatures between 60°C and 80°C. The nucleation and growth rates were shown to display Arrhenius kinetics, and the associated energy barriers were extracted for fibrils formed in bulk solution and at PS surfaces. These experiments showed that fibrils are nucleated more quickly in the presence of hydrophobic PS surfaces but that the corresponding fibril growth rates decrease. These observations are interpreted in terms of the differences in the attempt frequencies and energy barriers associated with the nucleation and growth of fibrils. They are also discussed in the context of differences in protein concentration, mobility, and conformational and colloidal stability that exist between insulin molecules in bulk solution and those that are localized at hydrophobic PS interfaces.

INTRODUCTION

The effects of surfaces and interfaces on the misfolding and subsequent aggregation of proteins are an important consideration in a number of biological and technologically important areas. For example the presence of an abundance of interfaces in vivo has resulted in a number of studies that were designed to assess the role played by model biological membranes and synthetic surfaces on the stability and aggregation properties of proteins (1–4). These separate studies showed that the presence of surfaces and interfaces can have a profound effect on aggregate development and that the growth rates and final aggregate morphologies are sensitive to the details of the surface chemistry of an interface (e.g., hydrophobicity, charge). However, many of these studies are semiquantitative in nature, and a more quantitative measure of the effects of surfaces and interfaces on the stability of proteins remains to be obtained.

Interfacial effects are particularly important for a number of protein conformational disease–related states that involve the aggregation of protein molecules into long fibrous structures called amyloid fibrils. These structures typically comprise linear aggregates of smaller protein units (e.g., monomers and dimers). They have diameters that are comparable to the dimensions of one or two protein molecules and can be many micrometers in length. These structures and their precursors have been linked to a number of diseases such as Alzheimer's disease, Huntington's disease, Parkinson's disease, type II diabetes, and Creutzfeldt-Jakob disease (5–8). In each case, the aggregates that are formed from different proteins have been found to exhibit very similar morphologies and to contain large amounts of chain-folded intermolecular β-sheet structures. This has recently led to the conclusion that amyloid fibril formation is a generic property of proteins (5–8). As a result, a significant amount of research has been carried out to try to develop an understanding of the molecular-level processes that result in their formation. The fact that these structures are usually formed in the presence of a wide range of biological interfaces also suggests that developing a greater knowledge of how protein/surface interactions affect the formation of these structures could help us to understand these processes.

Recent developments in the rapidly emerging field of nanotechnology also raise some important questions about the effects of surfaces and interfaces on the stability and aggregation properties of proteins and other biological macromolecules (9). Nanoscale materials and particles such as those being used in novel biosensing applications (10), immunological assays, and early gene gun–based delivery methods (11) have surface-to-volume ratios that are up to 10^8 times larger than bulk samples of the same materials. The surface-related effects that have already been reported in studies of protein aggregation (1–3) therefore have important

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implications for the nanotoxicology of a wide range of materials.

Protein aggregation is also of considerable interest to the pharmaceutical industry, where maintaining the stability of proteins at low pH is vital during the production and purification of protein-based drugs such as insulin (12–14). The storage of proteins is also an important factor in determining the shelf life of these materials. In particular, the effects of storing and processing proteins such as insulin in different containers are likely to affect their long-term stability.

In this article we describe an experimental study of aggregation and amyloid fibril formation in bovine pancreatic insulin (BPI) at elevated temperatures and low pH. Fourier transform infrared (FTIR) spectroscopy and attenuated total reflection (ATR) spectroscopy are used to monitor the aggregation/β-sheet-formation kinetics of this protein in bulk solution and on model hydrophobic polystyrene (PS) surfaces. The temperature dependence of the nucleation/lag times and aggregation rates associated with insulin fibril formation are used to determine the energy barriers associated with the nucleation and growth of these structures, respectively. Complementary techniques such as atomic force microscopy (AFM), dynamic light scattering (DLS), and thioflavin-T fluorescence assays were also used to monitor the growth of the protein aggregates. To the best of our knowledge, these experiments represent the first quantitative study of the effects of surfaces and interfaces on the aggregation properties of proteins.

EXPERIMENTAL PROCEDURES

Stock solutions of 0.025M NaCl and 0.1 M HCl (pH 1) were prepared in preboiled deuterium oxide (D_2O, Goss Scientific, Essex, UK). These solutions were then sealed in 15-ml sample vials and degassed by being placed in a water bath at ~85°C for 1 h. The solutions were then allowed to cool to room temperature. BPI (Sigma Aldrich, Gillingham, UK, mol wt = 5733, catalog No. I5500) was then dissolved in the solutions at a concentration of 20 mg·ml^{-1} (~3.5 mM). A single batch of BPI was used in all the experiments described below because batch-to-batch variations in the properties of insulin have been reported (15). The resulting protein solutions were then left at room temperature in sealed vials for a further 24 h to enable the complete hydrogen-deuterium exchange of labile hydrogens on the insulin molecules.

Fourier transform infrared spectroscopy

FTIR spectroscopy measurements were collected using a Varian FTS40 Pro spectrometer equipped with Resolutions Pro 4.0 software. Each averaged spectrum was collected using a spectral resolution of 4 cm^{-1}, and the time between the collection of the averaged spectra was 15 s. Deuterium oxide was used as the solvent in these experiments to avoid difficulties associated with the overlap of liquid water peaks with the amide I and amide II regions of the protein IR spectra. No spectral subtraction was performed on the infrared spectra. However, the baseline of the spectra tilted during the experiments as a result of variations in the intensity of the IR light source used in the spectrometer. This tilting was corrected for by subtracting a linear baseline, which was calculated using the flat regions of the spectra in the ranges 850–1100 cm^{-1} and 1750–2100 cm^{-1}.

Bulk solution IR spectroscopy measurements were performed over a range of temperatures from 60°C to 80°C using an electrically heated liquid transmission cell with zinc selenide (ZnSe) windows (Specac, Orpington, UK). One of the ZnSe windows was predrilled with two holes through its larger faces to allow the cell volume to be exchanged with fluid. Before the experiments, one side of each ZnSe window was spin coated with a 2 wt % solution of PS (M_w = 600 kDa, M_w/M_n = 1.09, Polymer Source) in toluene (spin speed 2500 rpm) and then annealed under vacuum at ~120°C to remove residual solvent. This resulted in the formation of an ~200-nm-thick PS film (as measured using atomic force microscopy). This was done to protect the windows from the acidic solutions used in the experiments. Acidic solutions are known to dissolve ZnSe, and Zn^{2+} ions have been shown to promote the onset of fibril formation in some proteins (16).

The two ZnSe windows were then placed together with their PS-coated surfaces separated by a 75-μm-thick poly (tetrafluoroethylene) (PTFE) spacer. This procedure was used to produce a small cavity with a 75-μm-thick path length for performing liquid transmission IR studies. The resulting assembly was then sealed around the edge using a highly viscous solution of PS in toluene. The windows and spacer were then annealed at ~100°C for 2 h to remove residual toluene and to seal the edges of the cell so that it would not leak when filled. The window assembly was then placed in the sample cell and loaded into the sample chamber of the FTIR spectrometer. Stainless steel tubing was then attached to the sample cell so that the volume of the cell could be exchanged from outside the FTIR sample chamber. The chamber was then purged with dry air.

The fully assembled liquid cell was then heated to the desired experimental temperature, which was monitored using a precalibrated t-type thermocouple buried in the metal body of the cell (close to the ZnSe windows). Deuterium oxide–based stock solution was then injected into the cell. A background spectrum was collected by averaging 100 individual spectra, and the stock solution was replaced with an identical solution containing 20 mg·ml^{-1} of BPI. Immediately after the injection of the protein solution, spectra were collected with a time resolution of 15 s. In each case 15 spectral scans were averaged per spectrum. All spectra were then ratioed to the spectrum obtained from the D_2O stock solution.

FTIR spectra of BPI adsorbed at PS surfaces were also collected using an ATR liquid cell (Specac). The ATR geometry uses the total internal reflection of IR radiation at a ZnSe/solution interface to produce an evanescent field that penetrates ~1 μm into the solution. Because the intensity of this field decays exponentially as a function of distance, the IR spectra that are obtained using this technique are extremely sensitive to material that is close to the interface.

Thin films (~200 nm) of PS were spin coated onto one side of a ZnSe ATR crystal using the same experimental parameters used to coat the ZnSe windows described above. The PS coated crystal was then annealed under vacuum at ~120°C to remove residual solvent and then allowed to cool to room temperature. The ATR crystal was then loaded into the ATR liquid cell and heated to the required temperature using a circulating water bath. The temperature in the cell was monitored using a thermocouple embedded in the ATR cell top plate that had been precalibrated against the measured temperature inside the cell. The sample cell was then filled with the D_2O-based stock solution and allowed to equilibrate before a background spectrum was collected by averaging 100 individual spectra. The solution was then replaced with an identical solution containing 20 mg·ml^{-1} BPI, and spectra were collected every 15 s (averaging 15 scans per spectrum).

Atomic force microscopy of BPI aggregates

BPI was dissolved in the D_2O-based stock solutions at a concentration of 20 mg·ml^{-1}. Bulk solution insulin fibrils were formed by incubating sealed vials of the resulting protein solution in a water bath at 60°C. The vials were then removed at regular time intervals and placed in an ice bucket to slow down the fibril growth rates and quench in the structure of the fibrils.

Surface-adsorbed fibrils were prepared on PS-coated single-crystal silicon wafers (Si, Compart Technology, [100], ~300 μm thick). An ~200-nm-thick PS film was spin coated on to the Si wafers from a 2 wt % solution in toluene and annealed at 120°C under vacuum. The resulting PS