Abstract
The effect of experimental conditions on the formation of structural intermediates and pathway of bovine insulin fibril formation was studied at pH 0.5 and 60°C. The relative amount of oligomeric intermediates observed during fibril formation was found to depend on both ionic strength and protein concentration. At a protein concentration of 1mg/mL, significant amounts of oligomeric intermediates including spherical assemblies and protofibrils were detected by AFM under the condition of 0.35 ionic strength. The oligomeric intermediates dissociated upon cooling for 10 days at 25°C. At 0.15 ionic strength, fibril formation proceeded without generation of oligomeric intermediates. Amorphous aggregates were observed prior to detection of well-defined fibrils under this condition. Increasing protein concentration from 1 to 2 mg/mL at 0.15 ionic strength or decreasing the protein concentration from 1 to 0.5 mg/mL at 0.35 ionic strength resulted in generation of similar low amounts of oligomeric intermediates. Fibril formation at 0.35 ionic strength and 1mg/mL of insulin was consistent with a nucleated conformational conversion mechanism, while fibril formation at 0.15 ionic strength and at the same protein concentration followed a nucleated polymerization mechanism. The results from this study show that ionic strength and protein concentration determined the relative quantities of structural intermediates formed during the early stages of fibril formation and the pathway of fibril assembly.

Keywords: Bovine insulin, fibril, protofibril, AFM

Introduction
Heating insulin in acidic solution or exposing to nonpolar surfaces such as air or plastic tubings predisposes the protein to rapidly form fibrils in vitro (1-5). Insulin fibrils are found to be unbranched, curved or linear, 3-4 nm in diameter with lengths reaching up to several microns (6-9). Dense crystal packing of the exposed hydrophobic surfaces between insulin molecules (7) results in the consistent size and compact shape of the basic structure of the insulin fibril called the protofilament (8). Several protofilaments can further aggregate laterally into bundles or twist into braids (6, 10), depending on the type of acidic media (i.e. HCl, H2SO4) and the process of fibril formation either by heat or agitation (11). Under certain experimental conditions, insulin can arrange spherically into formations called spherulites, which are composed of radially oriented fibrils around a core of less structured molecules (12, 13). Circular or ring shaped insulin fibrils have also
been observed after exposure to high pressure (14).

A simple model of insulin fibril formation has been proposed, which includes generation of a stable nucleus followed by growth of the nucleus into fibrils (12, 15). Growth or extension of the fibril is consistent with a first-order process (16) and is believed to propagate through a series of interactions between insulin molecules (8). Nuclei formation is hypothesized to be caused by the structural intermediates (7, 11, 16). These structural intermediates have been detected and characterized by NMR (17) and FTIR (10) and shown to retain much of the native insulin alpha-helical conformation. Recently, AFM was used to obtain detailed images of a unique insulin fibril assembly pathway in which short and thick seed-like forms appeared to function as lateral scaffolds for fibril growth (18).

Although significant progress has been made towards unraveling the mechanism of insulin fibrillation, much remains to be learned about the intermediates of the process and their role in protein fibril transformation (15). This study focuses on the relationship between experimental conditions (protein concentration, ionic strength), oligomeric intermediate formation, and fibril assembly pathway to further understand the mechanism of insulin fibril formation.

**Experimental Procedures**

**Protein and Reagents:** Bovine insulin, glutaraldehyde, NaCl, uranyl acetate, and thioflavin T (ThT) were purchased from Sigma-Aldrich (St Louis, MO). Bovine insulin was used without further purification. Reagent grade HCl was obtained from VWR International (Brisbane, CA). Mica disks and Formvar grids were purchased from Ted Pella, Inc. (Redding, CA).

**Preparation of Samples:** Stock solutions (0.5, 1 or 2 mg/mL) were prepared by dissolving bovine insulin into freshly-prepared 0.12 mM aqueous solutions of HCl (pH 0.5 at 60°C). The ionic strength was adjusted to either 0.15 or 0.35 using NaCl. Protein concentrations were determined by UV (276 nm) using an extinction coefficient of 1.0 for 1 mg/mL insulin (19). Aliquots of 0.5 mL protein solution were dispensed into 0.6 mL polypropylene centrifuge vials, sealed with Teflon tape, and incubated in 60°C water bath to initiate fibril formation. At different time points, samples were removed from the water bath and analyzed by DLS, AFM, TEM, and ThT fluorescence at room temperature. Oligomeric intermediates generated in samples heated from 0-60 minutes at 1 mg/mL and 0.35 ionic strength were then incubated for 10 days at 25°C and reanalyzed by DLS and ThT fluorescence spectroscopy.

**Atomic Force Microscopy (AFM):** Tapping mode AFM was conducted using a Nanoscope 3a Multimode system (Digital Instruments/Veeco, Santa Barbara, CA). Protein suspension (10µL) was deposited onto freshly cleaved mica and allowed to adsorb for one minute. The mica was rinsed with 200 µL of deionized water and dried with nitrogen gas. Tapping mode scans were taken in air, under ambient conditions at a frequency of 2.44 Hz using TESP tips (Digital Instruments). Images were processed and rendered using the Digital Instruments Nanoscope software. Images displayed as surface plots were contrast enhanced, low-pass frequency filtered and digitally zoomed from full size (600 nm) images.

**Transmission Electron Microscopy (TEM):** Electron micrographs were taken on Tecnai 12 transmission electron microscope (FEI, Hillsboro, Oregon) at 100-200 nm resolution. A 20 µL volume of sample was deposited onto Formvar coated grids and allowed to set for one minute before an equal volume of 0.5% (v/v) glutaraldehyde solution was added for an additional one minute. The grid was then rinsed with 4-5 drops of water and wicked dry with tissue before staining with 10 µL of 2% (w/v) of uranyl acetate for two minutes and finally wicked dry again with tissue.

**Dynamic Light Scattering (DLS):** DLS measurements were performed using a Dynaprobe 99 apparatus (Proterion Corp., Piscataway, NJ). Analyses of particle size distribution of insulin

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were determined using the solid sphere model and regularization method of the Dynamic V6 software. Protein samples were analyzed without filtration.

**Thioflavin T (ThT) Fluorescence Spectroscopy:** ThT fluorescence measurements were conducted using a Photon Technology International fluorimeter (Lawrenceville, NJ). Aliquots of 200 mL of thoroughly mixed sample suspensions were diluted into 2.5 mL of ThT (20 mM) phosphate buffer (pH 6.0). The samples were excited at 440 nm and fluorescence intensities at emission wavelength 480 nm were recorded.

**Results**

**Fibril Formation at High Ionic Strength:** Oligomeric intermediates (some in the shape of protofibrils) were produced in solution conditions of 1 mg/mL, 0.35 ionic strength, pH 0.5, and 60°C and then examined by AFM. Fig.1, shows a progression of structures formed during insulin fibrillation. The AFM image at time 0 shows a uniform layer of nonaggregated protein molecules. At 20 minutes, variable-sized spherical assemblies either attached or situated in proximity with one another are detected. A surface plot of the 20 minute sample (labeled 20 min*) showed structures that were similar to those designated as nucleation units found in the fibril formation of other amyloid proteins (20-23). The 60 minute sample contained short string-like assemblies. The surface plot of the 60 minute sample (labeled 60 min*) revealed various structures, including protofibrils and oblong globular forms fused with neighboring protein aggregates. By 110 minutes, a net of highly branched immature fibrils were evident. The 255 minute sample showed long, thin, unbranched fibrils emerging from the more slender but highly thickened mesh of fibrous insulin.

DLS was used to monitor the growth of oligomeric intermediates in solution. Prior to heat treatment (0 minute), light scattering showed a population with mean particle size 2.2 nm (Fig. 2). The small population peak at 0.1 nm was sometimes observed in pure water and so

**Fig. 1.** Temporal evolution of oligomeric intermediates and fibrils illustrated by AFM images (600 x 600 nm). Fibrils were generated by heating insulin (1 mg/mL, ionic strength 0.35, pH 0.5) at 60°C for the indicated times. Images labeled with asterisk symbol (*) are enlarged and contrast enhanced surface plots of species detected at the indicated times.
discarded. After 15 minutes of incubation, a second population with a mean size distribution of 14.4 nm was evident. This second population correlates temporally with the spherical assemblies observed by AFM in the 20 minutes sample (Fig. 1). At 60 minutes, the second population increased in dimension and scattering intensity to reach an average size of 37 nm. Large micron sized particles also appeared as a third population in these samples.

When the insulin concentration was reduced by half to 0.5 mg/mL while maintaining the ionic strength, pH, and temperature at 0.35, 0.5, and 60°C, protofibril-shaped oligomeric intermediates were observed by AFM. However, the amount of oligomeric intermediates generated at 60 minutes was significantly less than that generated from the 1 mg/mL sample incubated for the same length of time (compare Fig. 3 to the 60 min sample in Fig. 1). Even accounting for the decrease in protein concentration there was less than half the number of protofibrils present in a typical scanned image of the same size.

**Fibril Formation at Low Ionic Strength:**
Reducing the solution ionic strength to 0.15 at 1 mg/mL, pH 0.5 and 60°C resulted in the generation of structures different than the oligomeric intermediates detected at higher ionic strength after the 60 minutes lag time. Fig. 4B shows an AFM image of the 60 minute sample.

![Fig. 3. AFM image (600 x 600 nm) of protofibrils assembled under solution conditions of 0.5 mg/mL insulin, ionic strength 0.35, and pH 0.5 incubated at 60°C for 60 minutes.](image)

![Fig. 4. ThT fluorescence profile of insulin fibrillation for solution condition of 1 mg/mL insulin, ionic strength 0.15, pH 0.5, and 60°C (A). AFM (600 x 600 nm) image of amorphous aggregates detected at the 60 minutes lag time (B). TEM of fibrils generated at 150 minutes (C). Scale bar is 100 nm.](image)
As shown, nucleation units and protofibrils were not detected under these conditions. Instead, only variable sized amorphous aggregates are observed. The kinetics of insulin fibril formation as determined by ThT fluorescence showed an 80 minute lag time (Fig. 4A) for the formation of fibrils. Rapid fibril growth occurred over the following 20 minutes and reached a plateau by 100 minutes of incubation. TEM was used to confirm the presence of mature fibrils at the end of a 2.5 hour incubation period (Fig. 4C).

Increasing the protein concentration from 1.0 to 2.0 mg/mL resulted in the generation of protofibrillar oligomeric intermediates at 0.15 ionic strength, pH 0.5, and 60°C. Figures 5A and B shows AFM images of insulin protofibrils observed for samples incubated for 60 minutes. TEM confirmed the presence of abundant fibrils at the end of a 2 hour incubation period (Fig. 5C).

**Oligomeric Intermediate Causes Increase in ThT Fluorescence:** Oligomeric intermediates generated during the early stages of fibril formation caused increased ThT fluorescence. The elevated ThT fluorescence of these samples returned to baseline levels upon cooling to 25°C. ThT fluorescence was measured for samples which were shown to possess significant amounts of oligomeric intermediates as determined by AFM. Samples at 1 mg/mL of insulin, 0.35 ionic strength, and pH 0.5 were heated for 35 and 45 minutes and analyzed by ThT fluorescence. The black bars in Fig. 6 show a 10 and 75-fold increase in fluorescence intensity over baseline (value at time 0) determined for the 35 and 45 minute heated sample, respectively. The gray bars represent the fluorescence intensities remaining in the same samples after cooling for 10 days at 25°C. All samples exhibited near baseline fluorescence intensity values after cooling. No apparent visible precipitates were observed in the cooled samples, and DLS showed disappearance of the oligomeric intermediate size species from solution (Fig. 7). As expected, samples that were never heated (time 0) and stored for 10 days at 25°C showed only the insulin dimer population. Samples heated for 35 minutes and then stored for 10 days at 25°C showed dimeric insulin and micron sized particles. The samples heated for 45 minute and analyzed after storage showed only the dimeric insulin population.

![Fig. 5.](image1.jpg) Protofibrils and fibrils generated under solution condition 2 mg/mL, ionic strength 0.15, pH 0.5 and 60°C. (A) AFM image (600 x 600 nm) and (B) surface plot (200 x 200 nm) showing protofibrils present in samples heated for 60 minutes. (C) TEM image of fibrils generated after 2 hours of incubation.

Pathway of Bovine Insulin Fibril Formation
Discussion

Insulin remains a classic model protein for examining the fibril forming process under conditions of elevated temperature and acidic pH. The mechanism of fibril formation is primarily studied for strategies to prevent or reverse the process. Structural intermediates are investigated as possible targets for inhibition of fibril formation. Although intermediates have been found under a variety of conditions (10, 11, 16-18, 24-26) during the insulin fibril formation, its role in fibril formation remains controversial. This study was designed to explore the effect of solution ionic strength and protein concentration on the formation of structural intermediates during the early stages of insulin fibril formation and its significance in insulin fibrillation at elevated temperature and acidic pH.

Results from this study show that solution ionic strength played an important role in determining the type of structures generated during bovine insulin fibril formation. AFM revealed formation of oligomeric intermediates in shapes of spherical assemblies and protofibrils produced by conditions of elevated ionic strength as shown in Fig. 1 (solution condition: ionic strength 0.35, protein concentration 1 mg/mL, pH 0.5, and incubation temperature 60°C). These intermediates are visually similar to those reported for other amyloid proteins (20, 27).

Time sequence analysis of insulin aggregate formation by DLS and AFM provided insight to the pathway of insulin fibril formation and the role of the intermediates. Evidence from this study suggests that the oligomeric intermediates are on-pathway to fibril assembly. DLS detected a single 2.2 nm population consistent with the dimeric form of insulin in solution (16) for the unheated sample at time 0 (Fig. 2). Oligomeric intermediates were detected at 15 minutes as a single population averaging 14.4 nm giving a similar scattering intensity to that of the nonaggregated dimeric insulin population. Intermediates in the form of spherical assemblies were observed by AFM at 20 minutes (Fig. 1). These intermediates grew in size with

Fig. 6. Thioflavin T fluorescence detected after heating insulin at 60°C for the times indicated on x-axis (black bars). Gray bars show same analysis of the samples after further incubation at 25°C for 10 days. Solution conditions: 1 mg/mL, ionic strength 0.35, pH 0.5.

Fig. 7. Histograms of particle size distribution of samples heated at 60°C for the indicated times and then after further incubation at 25°C for 10 days. Solution conditions: 1 mg/mL, ionic strength 0.35, pH 0.5.
an average diameter of 37 nm and increased in relative number as determined by the greater scattering intensity of the oligomeric intermediates population compared to insulin dimers after 60 minutes of incubation. Abundant intermediates in the form of beaded protofibrils and elongated globular shapes were visualized by AFM after 60 minutes (Fig.1). We believe that the elongated globular structures are protofibrils that have attained increased bonding between neighboring spherical assemblies. The fibrous net-like material observed at 110 minute suggests that maturation and growth may occur partially through association of the shorter elongated globular forms. Realignment of protein molecules within the globular structure can explain the more slender appearance of the matured netted material. Maximal reorganization of the insulin molecules would result in the finer, more compact size of the fibril, as observed in the emerging fibrous mesh at 255 minutes (Fig. 1). Bovine insulin fibril assembly under this experimental condition is consistent with the nucleated conformational conversion mechanism proposed for other amyloid proteins (20, 28, 29).

Oligomeric intermediates in the shape of spherical assemblies for bovine insulin were also observed recently at the very early stages of fibril formation by Jansen et al. (18). Under the experimental conditions used by Jansen, the pathway of fibril assembly included the generation of short and thick seed-like structures providing lateral scaffolds for fibril growth (solution condition: 1 mg/mL, pH 1.6, 60°C).

In this study, the reversible characteristic of oligomeric intermediates was demonstrated using ThT fluorescence analysis, and supported by the size analysis using DSL and by visualization using AFM. Fig. 6 compares the ThT fluorescence intensities caused by oligomeric intermediates generated after heating samples up to 45 minutes at 60°C (black bars) with the samples analyzed after further incubation at 25°C for 10 days (gray bars). The fluorescence intensities detected in the samples after cooling returned to baseline values, while the oligomeric intermediate sized population disappeared as determined by DLS (Fig. 7). The dominant scattering intensity in all the cooled samples belonged to insulin dimers. The micron sized peak observed in the 35 minute sample can be attributed to amorphous aggregates that were formed or fibrils that were nucleated during the 10 day incubation period at 25°C. These results show that insulin oligomeric intermediates are distinguished from properly folded native insulin by their capacity to induce ThT fluorescence and from fibrils by their ability to reversibly dissociate. Induction of ThT fluorescence has also been reported for Alzheimer’s Ab (1-40) protein intermediates (30). Unlike fully assembled insulin fibrils, which do not readily dissociate (15) or decrease in ThT fluorescence intensity (31), insulin oligomeric intermediates were noted to reversibly dissociate with a consequent reduction in ThT fluorescence upon cooling to ambient temperature.

Under experimental conditions with lower ionic strength (solution condition: 0.15 ionic strength, 1 mg/mL, pH 0.5 and 60°C), bovine insulin fibril formation proceeded without generation of oligomeric intermediates. Examination of the species detected by AFM from the 60 minute sample revealed only amorphous aggregates (Fig.4B). The amorphous aggregates were not observed to increase ThT fluorescence as noted by the baseline value plotted in the fluorescence profile in Figure 4A. After a lag time of 70 minutes, the fluorescence intensity rapidly increased and reached a plateau at 100 minutes. This material consisted of long straight fibrils as confirmed by TEM (Fig. 4C). The sigmoid shape of the fluorescence curve shows classic fibril forming kinetics and is commonly associated with a nucleated polymerization mechanistic model of fibril assembly, in which rapid fibril growth occurs after successful formation of the nucleus (27). Bouchard and coworkers described a similar mechanism of bovine insulin fibril formation under a different set of experimental conditions (2 mM insulin, pD 2.67, 70°C) (10). Using TEM, they
detected clusters of amorphous aggregate at the early stages of fibril formation, which later disappeared with the concurrent increase in the density of fibrils. Although fibrils emerging from the amorphous clusters can sometimes be observed, no strong evidence supports the amorphous clusters to be intermediates on-pathway to fibril assembly.

To further understand the effects of protein concentration and ionic strength on fibril formation, we examined samples with various initial insulin concentrations and ionic strengths. Increasing the protein concentration from 1 to 2 mg/mL at lower solution ionic strength condition (0.15 ionic strength, pH 0.5, 60°C) resulted in production of a small amount of protofibrillar oligomeric intermediates detected in the 60 minutes sample (Fig. 5A and B). For this sample, TEM showed abundant fibrils after 2 hours of incubation (Fig. 5C). Decreasing the protein concentration from 1 to 0.5 mg/mL but at higher ionic solution strength (0.35 ionic strength, pH 0.5, 60°C) also generated similar amounts of oligomeric intermediates in the 60 minute sample (Fig. 2). These results show that the relative amount of oligomeric intermediates generated under the different experimental conditions is a function of both solution ionic strength and protein concentration. Higher protein concentration and higher ionic strength are more conducive to oligomeric intermediate formation. The effect of ionic strength on oligomeric intermediate formation can possibly be explained by a molten globule conformation of proteins in the presence of high salt concentrations (32-35). The propensity for molten globules to aggregate increasing the chances for oligomeric intermediates to form would explain the dependence on insulin protein concentration.

This study demonstrates the importance of environmental conditions on the structural intermediates that are formed during bovine insulin fibril formation. The different pathways of insulin fibril assembly observed in this study and those previously reported in other studies highly suggest a multi-pathway mechanism of insulin fibril formation that is dependent on solution conditions.

**Conclusion**

Ionic strength and protein concentration significantly affected the types of structures that were formed during the early stages of fibril formation, and subsequently determined the pathway of fibril assembly.

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**References**


