Insulin fibril nucleation: the role of prefibrillar aggregates

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Dynamic light scattering and Fourier Transform Infrared (FTIR) spectroscopy were used to study the formation of prefibrillar aggregates and fibrils of bovine pancreatic insulin (BPI) at 60^{o}C and at pH1. The kinetics of disintegration of the prefibrillar aggregates were also studied using these techniques following a quench to 25^oC. These experiments reveal that formation of prefibrillar aggregates is reversible under the solution conditions studied and show that it is possible to significantly alter the nucleation (lag) times associated with the onset of fibril growth in BPI solutions by varying the concentration of prefibrillar aggregates in solution. These results provide convincing evidence that less structured prefibrillar aggregates can act as fibril forming intermediates and can lower the energy barrier to fibril nucleation.

Introduction

Amyloid fibril formation is increasingly being considered to be a property of all globular proteins [1]. A recent article by Chiti and Dobson has cited 40 different human diseases that have been linked with the formation of these amyloid protein deposits [2]. However, fibrils are not the only type of protein aggregate that have been associated with these conditions. A number of recent studies have also reported the existence of smaller aggregates that precede fibril formation [3–9].

The importance of understanding the processes that are involved in the formation of the aggregates that precede fibril formation (prefibrillar aggregates) has been highlighted because of evidence that they (and not mature fibrils) may be the main cytotoxic species [6, 8–11]. It has been suggested that it is the misfolded nature of the proteins that leads to the cytotoxicity of these aggregates [8]. Misfolded globular proteins often have exposed hydrophobic residues that are usually hidden in the core of the molecules. The exposure of these residues could be responsible for abnormal interactions between the protein molecules and the constituents of a cell. As a result, the biochemical reactions that take place in and around the cell may be hindered by the presence of these aggregates [2].

Recent work by Yoshiike *et. al.* [12] has challenged these ideas. These authors propose that the lack of correlation that has been reported between the quantity of fibrils and their measured toxicity, could be caused by variations in the physicochemical properties of different fibril morphologies.

Smith *et. al.* studied insulin fibril formation and showed that small ($\sim$ 13 nm) spherical aggregates were formed prior to fibrils [13]. The insulin molecules that made up these prefibrillar aggregates were found to adopt a conformation containing a small amount of intermolecular β-sheet structure. Ahmad *et. al.* [7] have also provided experimental evidence for the existence of prefibrillar aggregates at several lower protein concentrations than those studied by Smith and coworkers. These studies indicate that prefibrillar aggregates are found under a variety of solution conditions that are capable of initiating fibril formation.

A key question that arises from these studies is related to whether prefibrillar aggregates are a necessary precursor for the nucleation of fibrils or whether they simply represent a competing aggregation pathway? Studies have been published that indicate that both of these scenarios can occur [4, 5, 14–16]. The task of elucidating the relationship between prefibrillar aggregates and protein fibril nucleation therefore remains to be of considerable importance.

In this article we describe an experimental study of the formation of prefibrillar aggregates and fibrils in solutions of bovine pancreatic insulin at elevated temperature and at pH 1. A combination of Fourier Transform Infrared (FTIR) spectroscopy and dynamic light scattering (DLS) measurements are used to study the formation of these aggregates at 60^oC. These techniques are also used to study the disintegration of prefibrillar aggregates following a rapid temperature quench from 60^oC to 25^oC. We show that it is possible to vary the concentration of prefibrillar aggregates in solution by varying the time following the quench. We also show that the nucleation (lag) time associated with the formation of fibrils depends upon the concentration of prefibrillar aggregates in solution. These experiments provide convincing evidence that the presence of non-fibrillar aggregates in solution can reduce the fibril nucleation (lag) times and promote

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the onset of insulin fibril formation.

Experimental

Protein Sample Preparation

Stock solutions containing 0.025M NaCl and 0.1M HCl (pH 1) were prepared in pre-boiled deuterium oxide (D_2O, Goss Scientific). These solutions were then sealed in 15 ml sample vials and degassed by placing them in a temperature controlled water bath at 85^oC for ~ 1 hour. The solutions were then allowed to cool to room temperature. Bovine Pancreatic Insulin (BPI, Sigma Aldrich, M_w =5733 Da, cat. no. I5500) was then dissolved in the solutions at a concentration of 20 mgml^{-1} ($\sim$ 3.5 mM). The resulting protein solutions were then left at room temperature in sealed vials for a further 24 hours to ensure that all the protein had dissolved.

Two sets of sample solutions (A and B) were then heated in vials on a Linkam THMS600 hotstage in a home built heater assembly. The vials were held at this temperature for 60 minutes. This procedure has been shown to result in the formation of prefibrillar aggregates in the protein solutions but no insulin fibrils are formed on these time scales [13]. The samples were then quenched rapidly to 25^oC by placing them in a temperature controlled water bath.

The two samples were then subjected to different thermal histories. This was done to determine how the β-sheet content of the protein solutions changed as a function of time following the temperature quench. In addition, these experiments were used to study the evolution of the size distributions and relative concentrations of protein aggregates at different temperatures (25^oC and 60^oC).

The first sample (A) was used immediately (within 5 minutes) to form fibrils by reheating the samples to 60^oC and allowing the aggregation process to reach completion by waiting a further 150 minutes [13]. The second sample (B) was maintained at a temperature of 25^oC for 5 days. This protein solution was then heated to 60^oC for 150 minutes and allowed to undergo further aggregation and fibril formation.

Dynamic Light Scattering

Small aliquots of BPI solution were taken from sample B at different times following the temperature quench from 60^oC to 25^oC and were rapidly transferred to a quartz cuvette. Dynamic light scattering measurements were performed using a Viscotek Dynamic light scattering apparatus (laser wavelength $633nm$). The temperature was maintained at 25^oC throughout the experiments and the size distributions of the aggregates and the total scattered light intensity were recorded as a function of time. Data were initially collected at 5 minute intervals for a period of 2 hours. Following this, data were collected at intervals of 60 minutes for a further 28 hours. Figure 1 shows examples of size distributions obtained at different times during these experiments.

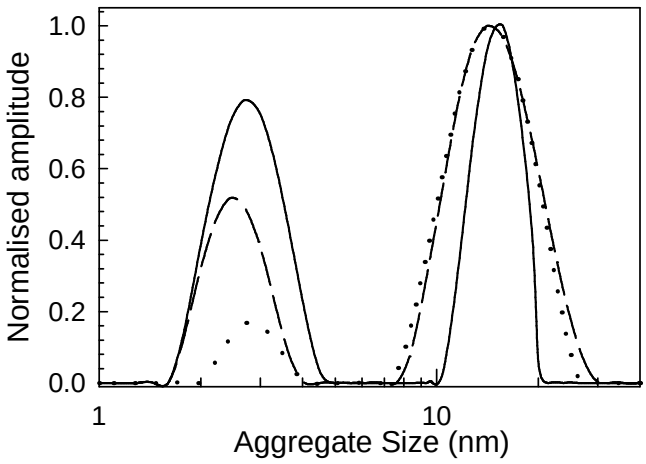

FIG. 1: Particle size distributions obtained from DLS studies of prefibrillar aggregates in solutions of BPI (20 mgml^{-1}, pH1) following a temperature quench from 60^oC to 25^oC. Data were collected at times of 150 minutes (dotted line), 500 minutes (dashed line) and 2940 minutes (solid line) after the quench. All size distributions are normalised in such a away that the size of the peak centered at $\sim$ 13 nm is equal to unity.

Fourier Transform Infrared Spectroscopy

Aliquots of solutions taken from sample B were also injected into an FTIR liquid transmission cell (75 micron path length, Specac, UK) following the temperature quench. The cell was preheated to 25^oC and infrared spectra were collected every 5 minutes (averaging 250 scans per spectrum, 4cm^{-1} resolution) for a period of 30 hours using a Varian FTS40 Pro spectrometer equipped with Resolutions Pro 4.0 software. All the spectra collected were ratioed to a background spectrum (averaging 250 scans per spectra) of the sample solution that did not contain BPI (see figure 2).

FTIR spectra were also collected from samples A and B and used to monitor changes in the β-sheet content of the BPI solutions during a second period of aggregation at 60^oC (i.e. when the samples were reheated following the temperature quench). Spectra were collected at 15 second intervals over a period of 150 minutes using a resolution of 4cm^{-1}. As shown by Smith *et. al.*, this provides a valid method for following the aggregation process in solutions of insulin and can be used to identify the different stages associated with prefibrillar aggregate and fibril formation [13].

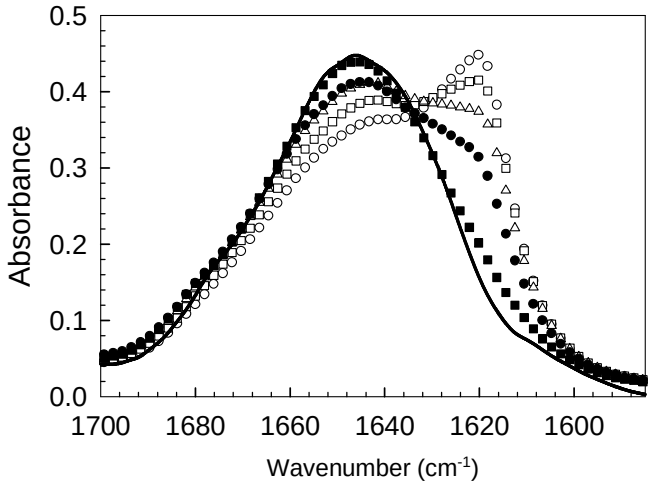

FIG. 2: FTIR spectra collected during the disintegration of prefibrillar aggregates in solutions of BPI (20 mgml^{-1}, pH1) following a temperature quench from 60^oC to 25^oC. Data are shown for spectra collected at times of 5 (◯), 60 (□), 150 (△), 500 (•) and 2100 minutes (■) following the quench. The solid line is a spectrum taken from freshly dissolved insulin at 25^oC.

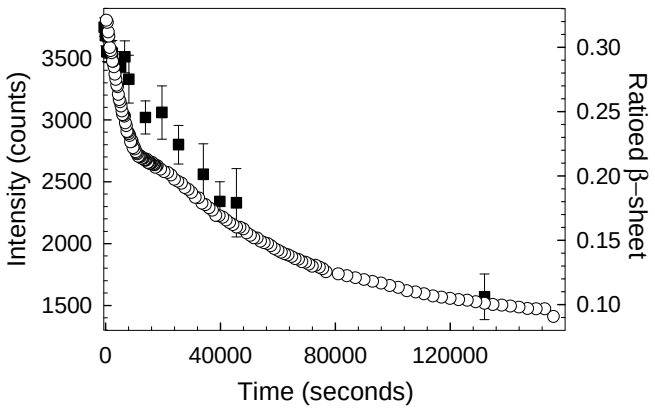

FIG. 3: Light scattering and FTIR studies of the disintegration of prefibrillar aggregates in solutions of BPI ((20 mgml^{-1},pH1)) following a temperature quench from 60^oC to 25^oC. Data are shown for the decay in the total light scattering intensity (■, DLS) and the area of the ratioed β-sheet peak (◯, FTIR, see text) as a function of time.

Results and Discussion

Figure 1 shows size distributions obtained from DLS measurements of the BPI solutions taken at different times following the quench from 60^oC to 25^oC. All the data shown in this figure is normalised in such a way that the intensity of the peak corresponding to the largest particle size observed is equal to one. This allows a qualitative comparison of the relative concentrations of each aggregate type to be made at each time. This figure shows that shortly after the quench, the BPI solutions contain a significant number of aggregates with a radius of $\sim$ 13 nm as well as a few structures that have a radius of $\sim$ 2-3 nm. These smaller structures are similar in size to those observed prior to any heating of the samples when insulin is dissolved under the experimental conditions used in this study (pH1, 0.025M NaCl). They are comparable to structures reported in other studies of insulin and are likely to arise as a result of the formation of insulin dimers and larger oligomeric structures that are known to occur under these solution conditions [7, 17].

The larger (13 nm) prefibrillar aggregates were observed to disintegrate into the $\sim$ 2-3 nm aggregates following the quench to 25^oC. This can be observed in figure 1 by the increase in the relative size of the peak corresponding to the 2-3 nm aggregates. It is worth noting that none of the DLS size distributions collected during these experiments revealed the presence of any aggregates of intermediate sizes.

The kinetics of aggregate disintegration can be followed more clearly by monitoring the total scattered intensity from all objects in solution as a function of time [5]. Figure 3 shows the time decay in the total scattering intensity measured in the DLS experiments following a quench from 60^oC to 25^oC. This figure shows that as time proceeds and the larger aggregates disintegrate into smaller structures, the total scattering intensity decreases. The observed decay in the scattering intensity can be interpreted using basic Rayleigh scattering theory. Bohren and Huffman [18] show that the intensity of light scattered from spherical particles in solution scales as $\sim cR^6$ (check this), where R and c are the radius and concentrations of the aggregates respectively. If we assume that both the large and small pre-fibrillar aggregates are spherical, then as the larger aggregates disintegrate, the concentration of smaller aggregates would be expected to increase by a factor of $N \sim \left(\frac{R_l}{R_s}\right)^3$. This means that to a first approximation the scattering intensity from a population comprised entirely of smaller aggregates (I_s) would be related to the intensity from a population of the larger aggregates (I_l) by the following equation

$$I_s = N I_l \left(\frac{R_s}{R_l}\right)^6 = I_l \left(\frac{R_s}{R_l}\right)^3 \qquad (1)$$

where R_s and R_l are the radii of the small and large aggregates respectively. Equation 1 confirms that the total scattering intensity is expected to decrease as the larger aggregates disintegrate into the smaller aggregate structures.

Figure 3 indicates that the decay in scattering intensity and hence the dissociation of the prefibrillar aggregates takes place on the time scale of days. Disintegration of the aggregates and changes in the secondary structure of the protein were also monitored using FTIR spectroscopy. Figure 2 shows FTIR spectra of the BPI solutions taken at times of 5, 60, 150, 500,

2100 minutes following the quench from 60^oC to 25^oC. This figure shows that as the large aggregates disintegrate, there is also a considerable change in the shape of the Amide I region of the IR spectrum of insulin. As discussed in a previous publication, this region can be accurately fitted to 3 Lorentzian curves corresponding to vibrational frequencies associated with random coil ($\sim 1645cm^{-1}$), α-helix ($\sim 1658cm^{-1}$) and β-sheet structures ($\sim 1610-1633cm^{-1}$). Fitting Lorentzian line shapes to these peaks enables the relative content of these structures within the protein molecules to be determined [13]. Figure 2 shows that there is a gradual decrease in the size of the β-sheet shoulder ($\sim$ 1620 cm^{-1}) of the amide I region of the protein spectra with time, following the quench to 25 ^oC. Qualitatively, this suggests that the β-sheet content of the protein molecules that comprise the $\sim$ 13 nm aggregates decreases as the aggregates disintegrate.

The peak fitting procedure mentioned above (and described in detail elsewhere [13]) was used to determine the variation in the β-sheet content of the protein as a function of time (see figure 3). The area of the β-sheet peak plotted in figure 3 was divided by the total area of the amide I region. The total area of the amide I region contains information about all of the secondary structures that exist within the protein molecules in solution and hence provides a quantitative measure of the amount of protein in the beam path. Dividing the area of the β-sheet peak by the total area of the amide I peak in this way therefore provides a measure of the amount of β-sheet per unit volume in the samples being studied [13].

Figure 3 shows that as the larger aggregates dissociate there is also a gradual decrease in β-sheet content of the protein. This figure also shows that the kinetics associated with the decrease in β-sheet content of BPI occur on similar time scales to the disintegration of the larger aggregates (as determined from the decrease in light scattering intensity). The ability of these larger (prefibrillar) aggregates to bind Thioflavin-T (ThT) dye molecules has also been used to show that there is initially some ordered β-sheet structure present within these aggregates. Smith et al [13] showed that prefibrillar aggregates of insulin are able to bind ThT in such a way as to produce a significant amount of fluorescence. The presence of a measurable ThT fluorescence intensity (above background levels) suggests that these aggregates contain repeat β-sheet units that are aligned in such a way that the ThT dye molecules can lie across several β strands associated with different protein molecules [19]. This indicates that the structures formed by the insulin molecules within these aggregates display some of the character of molecules that have been incorporated into mature fibrils [7].

As the prefibrillar aggregates disintegrate, the shape of the amide I peak (and hence the relative proportions of the different secondary structures) appears to revert to a state that is similar to that observed in freshly dissolved insulin (see figures 2 and 3). The correspondence between the timescales associated with the β-sheet content of BPI molecules and the dissociation of prefibrillar aggregates (measured by DLS) suggests that inter-molecular β-sheet plays an important role in determining the structure of the prefibrillar aggregates reported here (as has been established for the case of protein fibrils [17]). However, as shown previously , the β-sheet content per unit volume of the prefibrillar aggregates is smaller than that observed for insulin fibrils [13].

The ability to change the concentration of prefibrillar aggregates by varying the time following a temperature quench, provides a useful way of assessing their importance for fibril formation. Samples A and B were held at 25^oC for different periods of time (5 minutes and 5 days respectively) following a quench from 60^oC. These samples were then reheated to 60^oC and incubated for $\sim$ 150 minutes so that fibrils were allowed to form in solution. During this second incubation at 60^oC the β-sheet content of these samples was monitored using FTIR spectroscopy. The differences in prefibrillar aggregate concentrations between these two solutions were used to determine how prefibrillar aggregates influence the nucleation (lag) times associated with fibril formation.

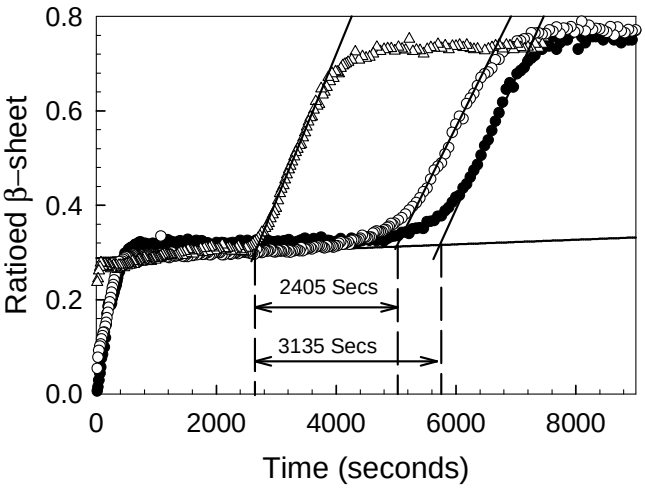

FIG. 4: Fibril nucleation studies in BPI solutions (20 mgml^{-1}, pH1) with different thermal histories. Data are shown for the ratioed β-sheet growth kinetics obtained from FTIR spectroscopy measurements of BPI solutions (see text). The data were collected from freshly dissolved BPI solutions that were incubated at 60^oC(•), as well as solutions that were heated to 60^oC for 60 minutes before being quenched to 25^oC and then a) reheated to 60^oC within 5 minutes (△) and b) held at 25^oC for 5 days before being reheated to 60^oC (◯). The solid lines shown in this figure mark the position of the plateau corresponding to the end of prefibrillar aggregate formation and the slopes of the regions corresponding to the period of fibril growth [13] respectively. The nucleation (lag) times for fibril growth can be determined from the times at which the fibril growth slopes intersect the plateau line.

Figure 4 shows the variation of the β-sheet content of

BPI molecules in samples A and B as a function of time. This figure also shows the variation in the β-sheet content of BPI taken from a sample of freshly dissolved protein that was incubated at 60^oC for the entire period shown on the plot (i.e. with no temperature quench to 25^oC). The β-sheet kinetics shown in this figure display the characteristic two phase aggregation kinetics that have been reported previously for BPI under these solution conditions [13]. The region in the β-sheet formation kinetics up to the end of the first plateau corresponds to changes associated with the formation and saturation of a population of $\sim$ 13 nm prefibrillar aggregates. The region of growth following this plateau has been attributed to changes in the β-sheet content of the protein associated with the formation of fibrils.

Figure 4 clearly shows that there is a difference in times associated with the onset of fibril nucleation for the two samples studied (samples A and B). The nucleation time for the sample containing a significant population of $\sim$ 13 nm prefibrillar aggregates (sample A) is significantly shorter ($\sim$ 2400 seconds) than the nucleation time observed in the samples where these aggregates have been partially dissolved by holding the protein solutions at 25^oC for 5 days (sample B). This indicates that the difference in time that both samples were kept at 25^oC (and hence the size of the population of prefibrillar aggregates), prior to being reheated, affects fibril nucleation times. These simple experiments lend support to the idea that prefibrillar aggregates can act to reduce the fibril lag times and lower the energy barrier associated with fibril nucleation.

The data shown for sample A in figure 4 clearly demonstrates that the fibril nucleation time is shortened by $\sim$ 60 minutes relative to that observed for a solution of freshly dissolved protein incubated at the same temperature. As this time difference is approximately the same as the time that sample set A was incubated prior to the quench, these results would seem to indicate that rapidly quenching the protein solutions and then immediately reheating them, preserves the population of the prefibrillar aggregates. As a result, the aggregation continues as if the quench had not occurred. However, when prefibrillar aggregates are allowed to dissociate (as in the case of sample B), the aggregation history of the system is effectively erased and the protein returns to a conformation which is predominantly α-helix and random coil (see figure 2). Whether the BPI molecules return to the same conformation as freshly dissolved insulin is not certain. However, the conformation contains the same relative proportions of different secondary structures observed for freshly dissolved BPI, making this a reasonable assumption.

The difference in fibril nucleation times observed between sample sets A and B was found to be $\sim$ 40 minutes. It might be expected that if the protein in sample B had reverted to its native conformation, the difference in nucleation times would correspond closely to the 60 minutes of heating that each sample experienced prior to the FTIR transmission measurements (as observed for freshly dissolved insulin). However, it should be noted that it is unlikely that the prefibrillar aggregates in sample set B would have completely dissociated, even after 5 days. If the concentration of prefibrillar aggregates relative to native protein in the BPI solutions was very small, they could still significantly increase the probability of a fibril nucleation event.

An alternative interpretation of these results could be given in terms of the differences in concentrations of the smaller protein structures (i.e. insulin monomers and dimers) that also exist between the two samples. However, this is unlikely to be the dominant factor in affecting the fibril nucleation times. We would expect that decreasing the concentrations of these structures would reduce the probability of them interacting in solution. If these structures were directly responsible for nucleating fibrils, this reduction in the collision probability would result in a longer fibril nucleation time being observed in the solutions with the lower concentrations of these smaller protein structures (i.e. in sample A). This is simply not observed in the experiments reported here.

Consideration of figure 4 shows that once prefibrillar aggregates have formed, a further $\sim$ 5000 seconds is required for fibril nucleation to take place. This can be deduced from the length of the plateau region observed in the first stage of the β-sheet formation kinetics shown for freshly dissolved insulin in figure 4. If prefibrillar aggregates are responsible for nucleus formation, as these experiments suggest, then why don't fibrils form immediately and what processes are occurring during the period corresponding to the plateau region in the kinetics?

Closer inspection of the plateau in the FTIR β-sheet kinetics in figure 4 reveals that the plateau is not completely flat, but exhibits a very slight increase throughout the time preceding fibril nucleation. This is also accompanied by a small shift (~ 4 cm^{-1}) in the position of the β-sheet peak to higher wave numbers (data not shown). These combined observations imply that the physical environment of the β-strands of the BPI molecules within the prefibrillar aggregates is changing during this time. This indicates that some local rearrangement of the BPI molecules is taking place within the aggregates and that some BPI molecules are reorganising into conformations that are more conducive to fibril formation.

A number of previous experimental studies have also shown that less well defined aggregates form in other protein systems. These studies have shown that structural rearrangements of the protein molecules can take place within the aggregates. These structural changes have been in a number of different proteins and have typically been shown to occur on time scales in excess of $\sim$ 10 hours [2, 4, 5, 10, 14].

Petty *et. al.* performed studies of the Syrian hamster prion peptide (H1) and used isotope modified IR spectroscopy to show that prior to fibril growth there is a necessary reorganisation and alignment of β-strands within prefibrillar aggregates [10]. They showed that the rearrangement and alignment of the β-strands in these

aggregates displayed an Arrhenius temperature dependence and that the structural changes lead to fibril nucleus formation. These combined results suggest that the time taken for realignment of β-strands within the prefibrillar aggregates is likely to be the rate limiting step for fibril nucleation in the current studies and may explain the associated lag time following the saturation of the population of prefibrillar aggregates.

Conclusions

Dynamic light scattering and FTIR spectroscopy were used to study the formation and disintegration of prefibrillar aggregates in solutions of bovine pancreatic insulin at 60^oC and pH1. The formation of the $\sim$ 13 nm radius prefibrillar aggregates was shown to be reversible and the concentration of these aggregates could be controlled by varying the amount of time the solutions were maintained at 25^oC following a temperature quench from 60^oC. This method was used to produce samples with different concentrations of prefibrillar aggregates. These experiments suggest that structural rearrangements occur in the protein molecules that comprise these aggregates. They also reveal that samples with larger concentrations of the $\sim$ 13 nm prefibrillar aggregates displayed shorter fibril nucleation times. These combined results provide convincing evidence that prefibrillar aggregates can act as fibril forming intermediates that lower the energy barrier and shorten the nucleation (lag) times associated with insulin fibril formation.

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