Structural Insight of Amyloidogenic Intermediates of Human Insulin

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ABSTRACT: Engaging Raman spectroscopy as a primary tool, we investigated the early events of insulin fibrillation and determined the structural content present in oligomer and protofibrils that are formed as intermediates in the fibril formation pathway. Insulin oligomer, as obtained upon incubation of zinc-free insulin at 60 °C, was mostly spherical in shape, with a diameter of 3–5 nm. Longer incubation produced "necklace"-like beaded protofibrillar assembly species. These intermediates eventually transformed into 5–8 nm thick fibers with smooth surface texture. A broad amide I band in the Raman spectrum of insulin monomer appeared at 1659 cm\(^{-1}\), with a shoulder band at 1676 cm\(^{-1}\). This signature suggested the presence of major helical and extended secondary structure of the protein backbone. In the oligomeric state, the protein maintained its helical imprint (~50%) and no substantial increment of the compact cross-\(\beta\)-sheet structure was observed. A nonamide helix signature band at 940 cm\(^{-1}\) was present in the oligomeric state, and it was weakened in the fibrillar structure. The 1-anilino-8-naphthalenesulfonate binding study strongly suggested that a collapse in the tertiary structure, not the major secondary structural realignment, was the dominant factor in the formation of oligomers. In the fibrillar state, the contents of helical and disordered secondary structures decreased significantly and the \(\beta\)-sheet amount increased to ~62%. The narrow amide I Raman band at 1674 cm\(^{-1}\) in the fibrillar state connoted the formation of vibrationally restricted highly organized \(\beta\)-sheet structure with quaternary realignment into steric-zipped species.

INTRODUCTION

Insulin is an essential protein that interacts with specific transmembrane cell surface receptors of muscle and adipose cells where it stimulates a complex cell signaling pathway, leading to the transport of glucose across the cell membrane and controls the glucose level in the bloodstream.\(^1\) It is produced in pancreatic \(\beta\)-cell,\(^2\) and its hexameric assembly formation and coordination to Zn\(^{2+}\) stabilizes insulin in cellular environment.\(^3\) It, however, exists as a mixture of hexameric, dimeric, and monomeric states in solution condition,\(^4\) and the thermal destabilization causes unwanted aggregation and malfunctioning. The aggregation and formation of amyloid-like fibril of insulin thus have long complicated its manufacture, storage, and clinical formulation.\(^5\)

It was postulated that the B-chain segment of insulin may be a crucial factor for insulin aggregation and fibrilization.\(^6\) The crystal structure reveals that insulin contains a large \(\alpha\)-helical structure consisting of S1 amino acid residues that are arranged into two polypeptide chains, A (21 residues) and B (30 residues). Three helical segments are formed by the residues, A2–A7, A13–A19, and B10–B19. Two of the helices are in the A-chain joined by a loop from A9 to A12 that brings the N- and C-termini together. The B-chain helix (B10–B19) is followed by a turn at the C-terminal end, and the N-terminal end of the B-chain is unstructured.\(^5\) Both the A and B chains of the molecule are linked together by two interchains (between A7 and B7 and between A20 and B19) and one intrachain disulfide bond (A6 and A11).\(^7\)

Several research groups hypothesized different mechanisms of insulin fiber formation\(^5\) and examined the aggregates under many circumstances, including low pH, different salt concentration, and hydrophobic interfaces that mimic physiological condition.\(^5\) Our earlier studies on insulin, mini-proinsulin, and domain swap mutants explored the secondary structural properties of globular and fibrillar forms using Raman spectroscopy.\(^8\) In later works, Hua et al. identified an early intermediate using solution-state NMR spectroscopy and reported that the N-terminal helix of both the A and B chain unfolds and detaches from the core.\(^5\) They also suggested that the C-terminal segment of B chain also unfolds to some extent.
but remains tethered to the core. Structural aspects of insulin aggregates were investigated several times,\textsuperscript{17,19−21} and the fibril showed a parallel intermolecular $\beta$-sheet rich secondary structure.\textsuperscript{22−25} Many of these studies suggested a three-phase process of matured fibril formation.\textsuperscript{26} During an initial lag phase, the protein may rearrange and position to start to form oligomeric intermediates and in the subsequent event, start stacking together forming elongated structures, commonly known as protofibrils. After this state, protofibrils can eventually pack, leading to the formation of twisted structures of mature amyloid fibrils. Thus, the formation of these ordered aggregated species is typically followed by a nucleation-dependent polymerization mechanism.\textsuperscript{27} It was reported that partially distorted non-native insulin monomers in oligomeric geometry play a key role in the kinetics of amyloid fiber formation\textsuperscript{28} and several studies show that the oligomeric species are the possible cause of cytotoxicity in amyloid disease in general.\textsuperscript{29} Therefore, uncovering the structural details of these species is very important to understand the nucleation-dependent fibril formation mechanism.\textsuperscript{30−32}

However, structural insights of the early intermediates and their conformational rearrangement are not well understood. Vestergaard et al. through small-angle X-ray scattering and computational modeling suggested that a helical structural nucleus could be the primary elongating unit of insulin amyloid fibrils.\textsuperscript{31} The shape and size of oligomers and their relative population in the lag phase control the rate of fibril formation. The current investigation attempted to provide proper coherent information about the conformational preferences and rate kinetics throughout the fibril formation pathway. We investigated the early stages of self-assembly and the changes in secondary structural or conformational rearrangement of insulin from native monomer to the fibrillar state by engaging Raman spectroscopy. Raman spectroscopy has been well used in the structural characterization of proteins and protein aggregates.\textsuperscript{33−35} The technique is highly sensitive to changes in conformation and bonding pattern, and the method is, therefore, particularly used to define the perturbation in the secondary and/or tertiary structural alignments in proteins in different assembly conditions. Here, we provided a definitive proof through Raman measurement that the fibrillation process of insulin involves the formation of a helix-rich intermediate in the lag phase; thereafter, in the maturation state, the helical conformation rapidly converted into the $\beta$-sheet rich structure.

**RESULTS AND DISCUSSION**

We investigated the early events of self-assembly, associated conformational rearrangement, and changes in the secondary structure of human insulin in the fibril formation pathway, engaging Raman spectroscopy as a primary tool. Figure 1 displays atomic force microscopy (AFM) images of different aggregate species formed in different time intervals upon incubation of human insulin (1.5 mg mL$^{-1}$ in 20 mM HCl with 50 mM NaCl, pH 1.8) for 3/4 h at 60 °C. For 60 min of incubation, it showed the formation of an oligomeric assembly structure, mostly spherical, with a diameter of 3−5 nm (Figure 1A). Pre-protofibrils were observed after $\sim$100 min of incubation.
Figure 2. Left panel shows the Raman spectra of insulin monomer, oligomer, and pre-proto-fibril in the frequency range of 800–1800 cm\(^{-1}\). The right panel presents the curve-fitting analysis of the extended amide I band. Panels (A) and (B), respectively, are the Raman spectra of insulin monomer and curve-fitting analysis to Raman amide I band of monomer. Panels (C) and (D) represent Raman spectra of oligomers and fitted bands, respectively. Raman spectra of pre-protofibrils and its amide I band fitting analysis are shown, respectively, in panels (E) and (F).

Table 1. Curve-Fitting Analysis of Amide I Raman Band Profile of Insulin and Its Aggregates, as Obtained at Different Time Points of Incubation\(^a\)

| time (min) | \(\alpha\)-helix | organized \(\beta\)-sheet | loose \(\beta\)-strand, PPII and disordered components | other disordered components (undefined components) |
|------------|-----------------|-------------------------|-----------------------------|---------------------------------|
| 0          | 1658 54 28      | 1676 9 21               | 1686 24 27                  | 1630 13 25                     |
| 60         | 1659 50 28      | 1676 8 19               | 1687 28 31                  | 1637 14 27                     |
| 120        | 1660 46 27      | 1675 12 22              | 1687 21 26                  | 1644 21 35                     |
| 135        | 1656 37 19      | 1673 29 20              | 1690 22 26                  | 1639 12 17                     |
| 160        | 1656 29 20      | 1675 41 19              | 1691 16 25                  | 1639 14 20                     |
| 180        | 1655 14 18      | 1674 62 18              | 1690 14 23                  | 1642 10 16                     |

\(^a\)Percentage of areas (%) and the bandwidth of each fitted component band are shown.
| monomer | oligomer | pre-proto fiber | proto fiber | premature fiber | fiber | modes of vibration |
|---------|----------|----------------|-------------|----------------|-------|-------------------|
| 850     | 831      | 831            | 831         | 830            | Tyr   | Tyr               |
| 854     | 852      | 852            | 852         | 850            | Tyr   | Cα-C stretching   |
| 898     | 898      | 890            | 890         | 885            | Cα-C stretching |
| 940, 949| 940, 949 | 940, 950       | 940, 949    | 940, 949       | Cα-C stretching |
| 962     | 964      | 960            | 960         | 960, 972       | skeletal β strand, Cα-C stretching |
| 1004    | 1004     | 1004           | 1004        | 1004           | Phe   |
| 1033    | 1033     | 1032           | 1032        | 1033           | Phe   |
| 1130    | 1133     | 1130           | 1135        | 1125           | Tyr   |
| 1156, 1177| 1156, 1178| 1156, 1175    | 1156, 1174  | 1153, 1170     | Phe, Tyr |
| 1205    | 1208     | 1206           | 1208        | 1209           | Phe   |
| 1249    | 1245     | 1250           | 1250        | 1254           | type II β-turn, strand, amide III |
| 1262, 1269| 1262, 1269| 1266          | 1267         | 1261, 1269     | α-helix, amide III  |
| 1277    | 1278     | 1276           | 1312        | 1312           | 2.5H, helix (extended β-strand), amide III |
| 1343    | 1341     | 1344           | 1345        | 1344           | lefthanded PPI, type III |
| 1421, 1449, 1465| 1423, 1449, 1463| 1422, 1449, 1463| 1419, 1449, 1464| 1419, 1444, 1454, 1463| CH2, CH3 deformation and scissoring |
| 1587    | 1588     | 1588           | 1589        | 1588           | Phe   |
| 1605    | 1607     | 1605           | 1604        | 1603           | Phe   |
| 1615    | 1617     | 1615           | 1616        | 1616           | Tyr   |
| 1659    | 1662     | 1662           | 1657        | 1656           | α-helix, amide I |
| 1676    | 1676     | 1676           | 1673        | 1676           | β-sheet, amide I |
incubation. The length and height of these pre-protofibrils varied up to several nanometers (Figure 1C). The surface morphology of pre-protofibrils was rough and indicated that no organized structures were yet formed. A more organized protofibrillar species formation was observed for 135 min of incubation, as shown in Figure 1D. The diameter of the aggregated species was 5–8 nm. Further incubation produced a threadlike amyloid fibril. Surface morphology showed a very smooth texture of fibrils and suggested an organized assembly of insulin monomers into a higher ordered assembly structure (Figure 1E,F).

Figure 2 shows the Raman spectra of insulin monomer, oligomer, pre-protofibril, protofibrils, and the late-state assembly structure of matured fibril, respectively. Table 1 contains the spectral assignments of characteristic Raman bands of the protein in different assembly conditions. The Raman spectrum of insulin monomer mainly consisted of a broad amide I band at 1659 cm\(^{-1}\) (Figure 2A).\(^{18}\) The bandwidth at half maxima (BWHM) for the band was \(\sim 49\) cm\(^{-1}\). This large bandwidth indicated the presence of multiple conformation states of the protein in its monomeric state. The band at 1659 cm\(^{-1}\) was an indication of major helical conformation along with a contribution from the disordered state, as marked at 1676 cm\(^{-1}\).\(^{18}\) The amide III region (1230–1300 cm\(^{-1}\)) was also enriched with predominant \(\alpha\)-helical marker bands (Figure 2A and Table 2).\(^{18,36–38}\) It was composed of a major shoulder band at 1262 cm\(^{-1}\) that marked the signature of pure helix.\(^{36,38}\) The signature of the extended helix/2.51-helix conformation similar to poly-L-glutamic acid conformation was embarked at 1268 and 1277 cm\(^{-1}\).\(^{39}\) The 1249 cm\(^{-1}\) shoulder was possibly due to mixed vibrational contributions from PPII and 3/10-helix conformation from the protein backbone structure (Table 2).\(^{38}\)

The Raman signature of insulin oligomer is shown in Figure 2C. It exhibited a broad amide I Raman band at 1661 cm\(^{-1}\). A
shoulder band appeared at 1676 cm\(^{-1}\) and quite resembled the band appearing for the monomeric state of the protein. It indicated the presence of structural heterogeneity in the oligomeric state similar to that of the monomer. We used a drop coating deposition Raman (DCDR) method to collect the Raman spectrum of monomer and aggregated species.\textsuperscript{40–42} In this process, 20–30 \(\mu\)L of the incubated sample was applied on a glass coverslip and air-dried. The methods helped to collect better quality Raman spectra of protein samples when solution-state experiments were difficult for several reasons. However, in the event of drying and evaporation, some conversion of monomer to oligomer formation may occur.\textsuperscript{42} Longer incubation of the protein solution produced pre-protofibril structures. The amide I band appeared at 1674 cm\(^{-1}\) and the BWHM was \(\sim 21\) cm\(^{-1}\) (Figure 3E,F). It indicated the formation of a major cross-\(\beta\)-sheet conformation of the protein in the fibrillar state. The narrow bandwidth indicated structural homogeneity and uniformity.\textsuperscript{34} A significant reduction in band intensity was observed for the \(\alpha\)-helical signature amide I band at 1657 cm\(^{-1}\).

The Raman band patterns and curve-fitting analysis to amide I band often provide ample information related to protein backbone conformation and its stability.\textsuperscript{18,36,37,45–46} Dong et al. and others performed initial Raman investigation on dimeric and hexameric insulin aggregates, measuring amide I band behavior in different sample conditions.\textsuperscript{34,47} Our laboratory also derived the structural content of amyloid aggregates of \(\alpha\)-peptide using the amide I curve-fitting protocol, as suggested by Dong et al. and Maiti et al.\textsuperscript{34,37} In our current investigation, curve fitting to the Raman amide I showed that \(\sim 54\%\) of residues in insulin monomer preferred \(\alpha\)-helical secondary structure and the component band was at 1658 cm\(^{-1}\) (Figure 2B and Table 1).\textsuperscript{18} A considerable amount (\(\sim 24\%\)) of residues remained in the extended/coiled structure, as indicated with a component band at 1686 cm\(^{-1}\).\textsuperscript{18} \(\beta\)-Sheet content was \(\sim 9\%\) and assigned to a component band at 1676 cm\(^{-1}\). Some contribution of the \(\beta\)-sheet secondary structure could be the result of drying, as the DCDR method was used to collect the Raman spectrum.\textsuperscript{42,48}

Figure 2D depicts the spectral deconvolution (curve fitting) of amide I Raman band of oligomers. In the oligomeric state, the protein maintained its helical imprint (50\%) and found no substantial increment of the core-\(\beta\)-sheet secondary structure. The deconvolution of the amide I band in the Raman spectra of the pre-protofibrillar state is shown in Figure 2E. We noticed some decrease in helical signature in the pre-protofibril state compared to that of the oligomer species. \(\alpha\)-Helix signature of the amide III band at 1262 cm\(^{-1}\) was somewhat decreased (Figure 2E and Table 2).\textsuperscript{36,38,39}

Formation of insulin protofibril was identified with the appearance of early \(\beta\)-sheet content as a signature by amide I band at 1673 cm\(^{-1}\) (Figure 3A,B and Table 2). In the protofibril species, the helical content decreased to 37\% (the component band at 1656 cm\(^{-1}\) ), whereas the \(\beta\)-sheet content increased to 29\% (the component band at 1673 cm\(^{-1}\), Table 1). The increment of \(\beta\)-sheet was due to the transformation of the helix and extended helix into \(\beta\)-conformational space (Table 1).\textsuperscript{18} Figure 3C,D shows the Raman signature of early fibrils (premature, incubation time 160 min). A considerable amount of loss in amide I helical marker band at 1657 cm\(^{-1}\) in comparison to the \(\beta\)-component band at 1675 cm\(^{-1}\) and thus a significant increase in \(\beta\)-sheet conformation was observed. A substantial shrinkage in the bandwidth was also observed (44 cm\(^{-1}\) for protofibril, 35 cm\(^{-1}\) for premature fiber). A significant gain in the \(\beta\)-sheet component was further established by the broad amide III \(\beta\)-sheet marker band at 1222 cm\(^{-1}\) (Figure 3C and Table 2).\textsuperscript{36,57,69}

We observed a very narrow and sharp vibrational amide I band at 1674 cm\(^{-1}\) (BWHM, 21 cm\(^{-1}\) ) in the fiber state of insulin, and a significant decrease in \(\alpha\)-helical signature amide I band intensity at 1657 cm\(^{-1}\) was observed. It indicated a major cross-\(\beta\)-sheet conformation of the protein in the fibrillar state (Figure 3C,E). In the fiber condition, the helical content was decreased to \(\sim 14\%\) (component band at 1655 cm\(^{-1}\) ), whereas the \(\beta\)-strand content was increased to \(\sim 62\%\) (component band at 1674 cm\(^{-1}\) ) and PPII/extended conformation was also decreased to \(\sim 14\%\) with a component band at 1690 cm\(^{-1}\) (Table 1). Amide III also showed a major band at 1222 cm\(^{-1}\) for \(\beta\)-sheet signature and a disappearance of the \(\alpha\)-helical band.
at 1260 cm$^{-1}$. The Raman bands at 1254 and 1274 cm$^{-1}$ were the marker for either molted helix or type III $\beta$-turn, respectively (Figure 3E and Table 2). Figure 4A graphically summarizes the overall changes in the secondary structural components with time (formation of different intermediate species), as obtained from Raman spectroscopic analysis.

In the process of insulin amyloid formation, a significant decrease of helical conformation was prominent, whereas extended conformations (PPII, extended helix) were continued until the formation of mature amyloid fiber (Table 1 and Figure 4A). The special $\alpha$-helical marker band that refers to the stretching vibration of $\mathrm{O}==\mathrm{C}==\mathrm{C}_n=\mathrm{H}$ linkage of the protein backbone near 1340–1345 cm$^{-1}$ was also clearly observed until the formation of fibrils (Figures 2, 3, and Table 2). The band intensity of the nonamide helix signature band at 940 cm$^{-1}$ also decreased substantially after the lag phase completion, as compared to that of the signal at 1434 cm$^{-1}$ region enlisted for the total CH$_2$ and CH$_3$ deformation and CH$_4$ scissoring of the protein itself (Figures 4B, 2, and 3). This again suggested that no major secondary structural change happened before the beginning of pre-protofibril formation.

Some monomers may transform into an oligomeric state in the DCDR method, and a strict comparison of structural components in monomer and oligomeric samples was not possible. However, our Raman analysis suggested that no major secondary structural changes occurred until the formation of protofibril species. The tertiary realignment could be the major force behind oligomer formation. The 1-anilino-8-naphthalene-sulfonate (ANS) binding study, as discussed later, suggested that a collapse in the tertiary structure and opening of hydrophobic surfaces occurred prior to the formation of protofibril species.

Thioflavin T (ThT) fluorescence assay is often performed to detect the formation of amyloid aggregates enriched with cross-$\beta$-sheet structure. ThT possibly bound to the cradle of cross-$\beta$-sheets of amyloid aggregates and attained a suitable conformation that increased its fluorescence yield. Therefore, ThT acts as a chemical probe and reports the formation of stable $\beta$-sheet structures in the process of aggregation of many proteins and peptides. To determine the content of the compact cross-$\beta$-sheet secondary structure in the different states of fibril formation, ThT fluorescence was measured at the different time points of incubation of the protein solution. Figure 5A displays the ThT fluorescence intensity at 485 nm in the presence of different insulin samples incubated for making the aggregates. The oligomers that were formed after 60 min of incubation did not cause major fluorescence enhancement of the ThT solution, suggesting no enhancement of cross-$\beta$-sheet structure in the oligomers. The fluorescence enhancement was started in the presence of protein samples incubated for $\sim$120 min. AFM confirmed that the species formed at this time point of incubation was pre-protofibril. The ThT fluorescence started increasing very rapidly in the presence of pre-protofibrils, and it was the maximum in the presence of fibril solution. Thus, the cross-$\beta$-sheet structural transition mainly happened in the late state of the aggregation process.

To further investigate the secondary structural transition of insulin in the fibril formation pathway, circular dichroism (CD) spectroscopic analysis was performed. Figure 5C shows the CD spectrum of insulin species at the different time points of incubation. The monomer at 0 min of incubation showed a CD pattern similar to that of native insulin in solution. In the oligomeric state, we observed no significant change in the CD signature. The negative CD signal at 208 nm and at 192 nm started to change in the pre-protofibril condition and indicated that the helical signature started to decrease from the pre-protofibril state. The CD spectra became quite broad (208–222 nm) in the protofibril condition and suggested the coexistence of several protein backbone conformations. The CD spectral signature of insulin fiber showed a strong negative band at 218 nm and defined the presence of the compact $\beta$-sheet structure. The deconvolution of very high quality CD
spectra often yields secondary structure components.\(^5\) However, for the aggregated samples, noise from scattering often disturbs spectrum quality and encounters unexpected errors. Therefore, the deconvolution of our CD spectra was avoided; it simply indicated that a major secondary structural change occurred after oligomer formation.

1-Anilino-8-naphthalene-sulfonate (ANS) binds to the exposed hydrophobic patches/surfaces of proteins when the surface opens up. The bound ANS produces enhanced fluorescence, and thus it enables to monitor the relative hydrophobic surface exposure during unfolding and refolding events, such as protein aggregation. To realize the tertiary collapse and exposure of the hydrophobic surfaces of structured insulin, we also performed ANS binding to the protein as monomer (0 min of incubation) and when it formed different aggregates in the process of fibril formation. A gradual increase in the ANS fluorescence in the presence of incubated insulin solution was observed (Figure S5B). However, the fluorescence dropped quickly in the presence of insulin samples incubated for more than 120 min and for the protein sample in the exponential growth phase of fibril formation. It appeared that the hydrophobic compact core of the protein slowly loosened and opened up, as time progressed, from its compact globular fold in the initial phase of aggregation. This indicated a tertiary collapse of the globular structure, without much changing its secondary conformation (as confirmed by CD, Raman, and ThT fluorescence results). The exposure of hydrophobic surfaces allows ANS molecule to access the hydrophobic surfaces. Interestingly, ANS fluorescence dropped suddenly for the samples incubated for more than 120 min when actual protofibril formation started. The protein molecules in amyloid fibrils are highly organized and attained a compact cross-\(\beta\)-sheet structure, as indicated in Raman and CD spectra. This again caused reduction of exposed hydrophobic surfaces as the molecules strongly associated to form fiber with a \(\beta\)-sheet structure. In the intermediate states (lag phase), the protein domain rearrangements and gradual melting/unwinding of the tertiary structure may occur and this may cause more and more exposure of hydrophobic surfaces. ANS could bind these exposed surfaces and enhance its fluorescence yield. These exposed hydrophobic surfaces eventually, however, associate strongly and get organized in the steric zipper mode\(^{54,55}\) to form cross-\(\beta\)-sheet rich amyloid-like insulin fiber.

**CONCLUSIONS**

Protein misfolding and aggregation processes are extensively studied leading edges in biochemistry as well as in molecular medicine for more than half a century. As such, protein aggregation and formation of the oligomeric state and fibrillar structure are linked to several pathologies, such as systemic amyloidosis, type II diabetes, and neurodegenerative disorders. However, in the trajectory of protein aggregation and fibril formation, the transition of protein monomer to oligomeric geometry is one of the most crucial events, from both the pharmacological and biochemical viewpoint. Our investigation on insulin fibrillization suggested that the tertiary structural collapse was a dominating event in the lag phase and subsequent oligomerization whereas minimal alteration occurred in its core secondary structure. Eventually, the exposed hydrophobic domains rapidly transformed into very organized cross-\(\beta\)-sheet rich fibrillar structure and in this late event of fibrillization, a remarkable secondary and tertiary realignment occurred in the polypeptide backbone.

**MATERIALS AND METHODS**

**Materials.** Zinc-free Insulin was purchased from Sigma Chemical Company (91077C). MilliQ water (Millipore Ltd., Bedford, MA) was used in all experiments, and the pH of the solution was controlled by using HCl. Thiouilain T and 1-anilinonaphthalene-8-sulfonic acid were obtained from Sigma. Stock solutions of thioflavin T (ThT) were prepared in MilliQ water, and concentration was checked using a molar extinction coefficient of 36 000 M\(^{-1}\) cm\(^{-1}\) at 412 nm. ThT solution was stored in dark at 4 °C.

**Preparation of insulin Aggregates.** Insulin solution for the aggregation study was prepared by dissolving zinc-free insulin in 20 mM HCl (pH 1.8) with 50 mM NaCl. The protein concentration was \(~1.5\) mg mL\(^{-1}\), and the final concentration was checked by absorption measurement at 276 nm.\(^7\) Each time, the freshly prepared protein solution was incubated for several hours at 60 °C without agitation and the formation of aggregate species due to incubation was followed by ThT assay and AFM using aliquots withdrawn at different time points of incubation. The integrity of the protein solution was checked by gel electrophoresis of the incubated samples. The gel image (Figure S2) indicated that some degradation and chemical modification might have occurred for longer incubation. The previous investigation also observed some changes in the chemical modification of insulin upon incubation for a longer time.\(^{16,56}\)

**AFM Imaging.** AFM images were recorded in Pico plus 5500 AFM (Agilent Technologies, Tempe, AZ) with a piezo scanner over the range of 9 \(\mu\)m. After incubation of the protein solution, aliquots were diluted 10–50 times and 10 \(\mu\)L of the diluted aliquots was drop casted onto a freshly cleaved mica surface. The sample was dried gently using air flow. Microfabricated silicon cantilevers of 225 \(\mu\)m length, with a nominal spring force constant of 21–98 N m\(^{-1}\) were used for imaging. The cantilever oscillation frequency was turned into the resonance frequency of 150–300 kHz. Images were processed by flattening using Pico view software (Molecular Imaging Corporation).

**Raman Spectroscopy.** A unique drop coating deposition Raman (DCDR) method was used to collect the Raman spectrum of monomer and aggregated species. This method was used by others in the field.\(^{30–42}\) Twenty to thirty microliters of the incubated insulin solution at different time points of incubation was placed on a glass coverslip and air-dried. Raman data were collected in the backscattering geometry with an STR Raman microscope (AIRIX Corp, Japan) under ambient condition (24 °C). A 633 nm He–Ne laser (model LGK 7665 P18, LASOS, Germany) with \(~1\) mW of laser power at the sample was used for excitation. The laser was focused onto the sample through the microscope (Olympus BX51M, Japan) using 50X objective, and Raman scattering was collected using a 500 mm focal length triple grating monochromator equipped with an air-cooled charge-coupled device detector. The spectra were recorded with a typical accumulation time of 100 s. The wave numbers of the Raman band were calibrated with the silica wafer focused under the 50X objective, and the spectral resolution was \(~1\) cm\(^{-1}\). The spectral data so obtained were processed with GRAMS/AI software.

**Curve Fitting of the Amide I Profile.** Amide I band of Raman spectrum is a characteristic band to investigate the changes in the secondary structure of protein.\(^{55,57,58}\) The band
fitting of the Raman amide I band was performed by using the Levenberg–Marquardt nonlinear least-squares process, as applied in the Curve Fit Ab routine of GRAMS/AI 9.02 software. The band region of 1575–1620 cm\(^{-1}\) was fitted assuming four asymmetric component bands that represented different structural conformations of insulin: α-helical band at 1655 cm\(^{-1}\) with spectral window (1650–1660 cm\(^{-1}\)), β-sheet component band near 1673 cm\(^{-1}\) (1670–1675 cm\(^{-1}\)), polyproline II or loose β strands and disordered structure near 1685 cm\(^{-1}\) (1680–1690 cm\(^{-1}\)), and disordered structure or vibronic coupling band near 1637 cm\(^{-1}\) (1630–1645 cm\(^{-1}\)). During amide I band fitting, three different bands at ~1585, ~1604, and ~1615 cm\(^{-1}\) for the ring vibrational mode of phenylalanine and tyrosine were also included.\(^{37,43}\) Component bands were fitted by 15–40 cm\(^{-1}\) bandwidth features; Gaussian and Lorentzian functions were allowed for homogenous and heterogeneous broadening. The standard error for peak positions and peak widths were <5 cm\(^{-1}\) for well characteristic components; this typically introduced an uncertainty of ~10% in the measurement of area under each fitted curve.

Thioflavin T (ThT) Fluorescence Spectroscopy Assay. ThT fluorescence assays were performed to investigate insulin amyloid fibrillation. At room temperature, 10 μL of the incubated insulin sample at the different time points of incubation was individually added to 700 μL of ThT solution (25 μM) and mixed properly. The solution was excited at 440 nm and emission was recorded at 485 nm on a VARIAN CARY Eclipse fluorescence spectrometer using a 1 cm path length quartz cuvette. The integration time and slit widths were fixed at 1 s and 5 nm, respectively. ThT in buffer without protein was used as a baseline.

Circular Dichroism (CD) Spectroscopy. A JASCO J-815 spectropolarimeter (Jasco) equipped with a Peltier temperature control unit which was set to 25 °C with an accuracy of ±0.1 °C was used to measure the protein’s secondary structure at different times of incubation. The data acquisition interval time was 2 s. Ten microliters of the incubated protein solution each at different time points of incubation; and Table S1, Raman vibrational bands of insulin monomer as air-dried, solid powder, and in the aqueous solution state (PDF)

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Notes
The authors declare no competing financial interest.

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