High Pressure FT-IR Spectroscopic Study on the Secondary Structural Changes in Insulin Amyloid Fibril and Aggregate

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Summary

We have examined the pressure-induced changes in secondary structure for the two kinds of insulin aggregate, amyloid fibril and reduction-induced aggregate, by using FT-IR spectroscopy. The parallel β-sheet for the amyloid fibril is not unfolded, but dramatically distorted with increasing pressure up to 1.0 GPa. This structural rearrangement is elastic, and does not cause the backbone amide protons involved in parallel β-sheet to expose to aqueous medium. The dissociation of the amyloid fibril into its monomer or oligomer is not likely to be promoted by application of high pressure. On the other hand, the antiparallel β-sheet for the reduction-induced aggregate remains distinct at 1.3 GPa. The whole construction is quite rigid in spite of the same origin (i.e. insulin) as the amyloid fibril is formed. The difference in structural elasticity implies that the larger size or the more number of cavities is included in the internal regions of insulin amyloid fibril. The tertiary interactions between side chains for the amyloid fibril are not likely to be as tight as that for the reduction-induced aggregate and native form.

Introduction

The stability of proteins in aqueous medium depends on solvent composition, temperature, and pressure. Not only under conditions favoring the denatured state, but also even under physiological (i.e. strongly native) conditions, proteins aggregate frequently. A number of human diseases, known as amyloidosis, originate from the deposition of stable protein aggregates with highly ordered structures. Recent studies suggest that the assembly into amyloid fibrils is an intrinsic property for peptides and proteins (Fändrich et al., 2001; Dobson, 2003). However, the intra- and intermolecular interactions which promote the formation of protein aggregates and amyloid fibrils and stabilize their amorphous or organized structures are not understood in detail. It is the absolute advantage that FT-IR spectroscopy can be used to study the secondary structure of proteins in the insoluble aggregated state, which is induced by both native (e.g. salted out) and denatured (e.g. thermally-, chemically-, or pressure-denatured) form, as well as in the soluble native and denatured state. Upon the thermally-induced denaturation of many proteins, the two amide(I/I′) component bands characteristic of their aggregates with intermolecular antiparallel β-sheet structures are frequently observed at 1610-1620 cm⁻¹ and 1680-1690 cm⁻¹ (Ismail et al., 1992; Naumann et al., 1993; Holzbaur et al., 1996; Dong et al., 2000; Militello et al., 2004). It has been also revealed by the amide(I) band that parallel or antiparallel β-sheet structures are
predominant in the amyloid fibrils originating from many different proteins, peptides and homogeneous polypeptides (Bouchard et al., 2000; Fändrich et al., 2002; Goers et al., 2002; López de la Paz et al., 2002; Fändrich et al., 2003; Kammerer et al., 2004) In this study, we have revealed the pressure-induced changes in secondary structure for the two different kinds of insulin aggregate by using FT-IR spectroscopy. On the basis of the difference in pressure effects between insulin amyloid fibril and reduction-induced insulin aggregate, the interactions which stabilize each organized insulin aggregate are examined.

**Experimental Methods**

**Preparation for Insulin Amyloid Fibril**

Insulin from bovine pancreas (Sigma-Aldrich) was dissolved in 100 mM NaCl aqueous solution adjusted to pH 2.0 with HCl. The concentration of insulin was 2 mg/ml. After the filtration by cellulose acetate membrane with 0.2 µm pore size (Toyo Roshi), the insulin solution was incubated at 60°C for 3 days with gentle agitation. The formation of amyloid fibril was checked by thioflavin T assay.

**Preparation for Reduction-Induced Insulin Aggregate**

The reduction of insulin was catalyzed by bovine protein disulfide isomerase (PDI), accompanying the aggregate formation. On the basis of the procedure described by Lambert and Freedman (Lambert and Freedman 1983), the proceeded reaction medium finally contained 30 nM PDI, 8mM glutathione, 0.4 mM NADPH, 1 unit of glutathione reductase, 30 µM insulin, and 0.2 M sodium phosphate buffer (pH 7.5) containing 5mM EDTA. The formation of reduction-induced insulin aggregate was completed in several hours at 25°C.

**Preparation for Native Insulin Solution**

Insulin was dissolved in D₂O (Euriso-Top) adjusted to pH 2.0 with DCl (Sigma-Aldrich). The pH was directly read from a pH meter without the adjustment for isotope effects. The concentration of insulin was 30 mg/ml. After the incubation at room temperature overnight in order to deuterate the exchangeable backbone amide protons in insulin, the solution was used without further treatments for FT-IR measurements.

**High Pressure FT-IR Measurements**

The reaction mixture containing the amyloid fibril or reduction-induced aggregate of insulin was centrifuged at 10,000 g for 10 minutes at 5200 g or for 5 minutes at 2000 g, respectively. The concentrated aggregate and fibril were resuspended in D₂O. These processes were repeated four times, except that 50 mM Tris-DCl D₂O buffer (pD 7.6) is used instead of D₂O at the last two times. Then, the suspensions were incubated at 4°C overnight.

All samples for FT-IR measurements placed together with a small amount of powdered barium sulfate (BaSO₄) in a 1.0 mm diameter hole of 0.1 mm thick stainless-steel (SUS 301) or hastelloy C-276 gasket mounted on a home-made diamond anvil cell. The barium sulfate was used as an internal pressure calibrant (Wong and Moffat, 1989). Infrared spectra of the native solution or the suspensions were recorded at 25°C using a Jasco 680 plus Fourier transform infrared spectrometer equipped with a liquid-nitrogen cooled MCT detector. The infrared beam was condensed by a zinc selenide lens system onto the sample in the diamond anvil cell. Temperature was controlled within 0.2°C by means of a jacket of circulating water. For each spectrum, 256 interferograms were co-added, applied a zero-filling, and Fourier transformed to give a spectral resolution of 2 cm⁻¹ with a cosine apodization function. 20 minutes were allowed to equilibrate the suspensions at the chosen...
pressure prior to each infrared measurement which itself takes 5 minutes. Pressure was increased at the average rate of about 200 MPa/h. In order to eliminate spectral contributions of atmospheric water vapor, the spectrometer and sample chamber were continuously evacuated and purged with dry air, respectively. The spectra of only D$_2$O or D$_2$O buffer were recorded in the same cell and under the same instrument conditions as the native solution or suspension spectra. The infrared spectra for native insulin, insulin amyloid fibril and reduction-induced insulin aggregate were obtained by digitally subtracting the appropriate D$_2$O or D$_2$O buffer spectrum from the spectrum of native solution, fibril, or aggregate suspension. Then, the bands originating from water vapor were subtracted until the absorption-free region of the amide(I') band above 1700 cm$^{-1}$ was featureless. The second-derivative spectra were generated by using a 19-data-point (approximately 9 cm$^{-1}$) Savitzky-Golay derivative function available from Jasco’s software, Spectra Manager for Windows 95/NT ver. 1.53.02.

Results and Discussion

Secondary Structure of Native Insulin, Insulin Amyloid Fibril, and Reduction-Induced Insulin Aggregate

The amide(I') component bands at 1656 cm$^{-1}$ for native insulin (Figure 1A), 1628 cm$^{-1}$ for insulin amyloid fibril (Figure 1B), and 1621 and 1694 cm$^{-1}$ for reduction-induced insulin aggregate (Figure 1C) are assigned to the peptide segments in the $\alpha$-helix, intermolecular parallel $\beta$-sheet and intermolecular antiparallel $\beta$-sheet structure, respectively (Surewicz et al., 1993; Barth and Zscherp, 2002). The predominant secondary structure is significantly different from one another, also suggesting the changes in the manner of tertiary interactions between side chains to stabilize the ordered structure of native insulin and two different kinds of insulin aggregate.

Figure 1: Original (blue line) and 2nd-derivative (red line) infrared spectra in the amide(I') region for native insulin (A), insulin amyloid fibril (B), and reduction-induced insulin aggregate (C). These spectra were measured at 25 °C and ambient pressure (0.1 MPa).
Pressure Effects on Insulin Amyloid Fibril

Application of high pressure to insulin amyloid fibril induces the dramatic decrease in intensity of the amide(I') component band attributed to the intermolecular parallel β-sheet structure with a low-frequency shift (Figure 2A and 2C). The amide(I') band contour indicates that the amyloid fibril has some residual secondary structure elements even above 1.0 GPa (Takeda et al., 1995). Since the band intensity in second-derivative spectrum is very sensitive to not only the band intensity but also the band width in original spectrum, however, it is difficult to determine the content of the intermolecular parallel β-sheet. Undoubtedly, the parallel β-sheet structure included in insulin amyloid fibril is significantly distorted under high pressure with a high reversibility. After pressure released to 0.1 MPa, the amide(I') component band attributed to the intermolecular parallel β-sheet structure is completely recovered without further hydrogen-deuterium (H-D) exchange of its involved backbone amide protons (Figure 2B). The H-D exchange measurements by mass spectroscopy have revealed that many of the labile protons in oligomeric insulin species are strongly protected under the conditions where insulin favorably forms amyloid fibrils (Nettleton et al., 2000). Therefore, no backbone amide protons involved in the amyloid parallel β-sheet expose to aqueous medium with increasing pressure. The dissociation of insulin amyloid fibril into its monomer or oligomer is not likely to be promoted by application of high pressure. Consequently, the intermolecular parallel β-sheet structure for insulin amyloid fibril possesses elastic construction.

Figure 2: Pressure effects on insulin amyloid fibril. (A) The changes in 2nd-derivative amide(I') band with increasing pressure. (B) The 2nd-derivative amide(I') band at 0.1 MPa before pressurization, at 1020 MPa, and after pressure released to 0.1 MPa. (C) Pressure dependence on the frequency (square) and relative intensity (triangle) of amide(I') component band attributed to the intermolecular parallel β-sheet structure. Green square was measured after pressure released to 0.1 MPa.
**Pressure Effects on Reduction-Induced Insulin Aggregate**

The intermolecular antiparallel β-sheet structure included in reduction-induced insulin aggregate remains almost intact even at 1.3 GPa, which does not cause its involved backbone amide protons to be exposed to aqueous medium under high pressure (Figure 3A, 3B, and 3C). However, the minor structural rearrangements are likely to be induced. The frequency splitting between the two amide(I') component bands attributed to the intermolecular antiparallel β-sheet structure increases with increasing pressure (Figure 3C), indicating that the reduction in distance between adjacent β-strands and/or the increase in planar β-sheets (Barth and Zscherp, 2002). It is evident that reduction-induced insulin aggregate is not also dissociated by application of high pressure. The intermolecular antiparallel β-sheet structure for reduction-induced insulin aggregate possesses relatively rigid construction.

![Figure 3](image)

**Structural Elasticity for Insulin Amyloid Fibril**

The pressure response to secondary structure for insulin amyloid fibril is significantly different from that for reduction-induced insulin aggregate in spite of the same origin. Whereas the parallel β-sheet for amyloid fibril is elastic construction, the antiparallel β-sheet...
for reduction-induced aggregate is rigid one. This difference in structural elasticity implies that the larger size or the more number of cavities is involved in the internal regions of amyloid fibril (Silva et al., 2001). The tertiary interactions between side chains for the amyloid fibril are not likely to be as tight as that for the reduction-induced aggregate and native form.

**Conclusion**

The parallel $\beta$-sheet for insulin amyloid fibril possesses more elastic construction than the antiparallel $\beta$-sheet for reduction-induced insulin aggregate in spite of the same origin. This difference in structural elasticity implies that the larger size or the more number of cavities is included in the internal regions of amyloid fibril. The looser tertiary interactions between side chains might be less effective in maintaining the secondary structure of insulin amyloid fibril against application of high pressure. The high pressure FT-IR spectroscopic study on protein aggregates and amyloid fibrils should provide the unique and important clues to understand the intra- and intermolecular interactions which form and stabilize their organized structures.

**References**


