FOR THE RECORD

Insulin forms amyloid in a strain-dependent manner: An FT-IR spectroscopic study

WOJCIECH DZWOLAK,¹ VYTAUTAS SMIRNOVAS,¹ RALF JANSEN,² AND ROLAND WINTER²

¹High Pressure Research Center, Polish Academy of Sciences, 01-142 Warsaw, Poland

²University of Dortmund, Department of Chemistry, Physical Chemistry I, D-44227 Dortmund, Germany

(RECEIVED December 27, 2003; FINAL REVISION March 9, 2004; ACCEPTED March 25, 2004)

Abstract

The presence of 20% (v/v) ethanol triggers growth of insulin amyloid with distinct infrared spectroscopic features, compared with the fibrils obtained under ambient conditions. Here we report that the two insulin amyloid types behave in the prion strain-like manner regarding seeding specificity and ability of the self-propagating conformational template to overrule unfavorable environmental factors and maintain the initial folding pattern. The type of the original seed has been shown to prevail over cosolvent effects and determines spectral position and width of the amide I' infrared band of the heterogeneously seeded amyloid. These findings imply that "strains" may be a common generic trait of amyloids.

Keywords: insulin; amyloid; prion strains; cross-seeding; protein aggregation

Amyloids, which used to be associated with a limited number of pathological conditions, such as Alzheimer disease or the bovine spongiform encephalopathy (BSE)-related Creutzfeldt-Jakob disease, are now thought to represent a common generic feature of proteins as polymers (Fandrich and Dobson 2002). Although comprehensive mechanisms of protein aggregation and amyloidogenesis remain elusive, the problem draws a lot of attention because of its paramount implications in life sciences (Cohen and Kelly 2003).

The perplexing observation that a single amino acid sequence of the prion protein propagating in genetically identical hosts may still evoke several distinct phenotypes (both in terms of clinical symptoms and physicochemical properties) of the prion—the so-called prion strains—was initially received as a challenge to the postulated protein-only eti-

ology of the prion disease. The claim as to the common generic character of amyloids as polymers led us to inquire whether the prion strains themselves stem from an analogous common generic trait of amyloids, including those unassociated with prion diseases. To test this hypothesis, we had to find a suitable amyloidogenic protein genetically unrelated to strain-forming prion precursors.

Insulin is genetically distant from proteins usually associated with pathological aggregation *in vivo*, yet it easily forms amyloid *in vitro*. The wealth of biochemical and structural data has made it an excellent model for amyloid studies (see Nielsen et al. 2001). The insulin aggregation is an energetically self-sustaining process (Dzwolak et al. 2003), which enables carrying out amyloid-seeding experiments at ambient temperature. Seeding amyloid instead of inducing it through *en masse* destabilization of the native protein (e.g., by prolonged heating) is a more adequate model of an *in vivo* process. Furthermore, seeding under non-denaturing conditions effectively prevents spontaneous formation of templates other than the originally added seeds themselves. Morphology and infrared spectra of insulin fibrils have been shown to depend on the presence of cosolvents (Nielsen et al. 2001). In this work, two distinct

Reprint requests to: Wojciech Dzwolak, High Pressure Research Center, Polish Academy of Sciences, Sokolowska 29/37, 01-142 Warsaw, Poland; e-mail: wdzwolak@unipress.waw.pl; fax: 48-22-632-42-18.

Abbreviations: FT-IR, Fourier transform infrared spectroscopy; AFM, atomic force microscopy; EtOD, d₁-deuterated ethanol; HHBW, bandwidth at half height; BSE, bovine spongiform encephalopathy.

Article published online ahead of print. Article and publication date are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.03607204>.

types of insulin amyloid formed at low pD and in the presence or absence of 20% (v/v) ethanol were used as seeds to trigger second generations of fibrils in identical and cross-altered insulin solutions.

Fourier transform infrared spectroscopy (FT-IR) spectroscopy is a sensitive and commonly used technique of monitoring α -helix-to- β -sheet transition, which accompanies aggregation of α -helical proteins (Dzwolak et al. 2002, 2003). Upon aggregation of insulin, the secondary structure-sensitive amide I' vibrational band shifts from $\sim 1655\text{ cm}^{-1}$, the wavenumber typical for α -helical proteins, to $\sim 1627\text{ cm}^{-1}$. This region is assigned to parallel β -sheet, which is the dominating secondary fold in amyloid fibrils. The method has proved very revealing in characterization of prion strains (Caughey et al. 1998), mutant Alzheimer fibrils (Fabian et al. 1993), and insulin amyloids (Bouchard et al. 2000; Nielsen et al. 2001).

Results

The seed-induced aggregation of bovine insulin at pD = 1.9 and 25°C was followed by time-resolved FT-IR spectroscopy (Fig. 1A). The infrared spectra show a gradual shift of the amide I' band to 1625 (in acidified D_2O) or 1622 (acidified D_2O with 20% (v/v) ethanol) cm^{-1} , accompanied by a marked narrowing of the peak, both of which reflect the complete transition of the native structure into nonnative aggregated β -strands. The amyloid character of final insulin aggregates was confirmed by atomic force microscopy (AFM) scans showing typical, predominantly straight, unbranched fibrils (Fig. 1B).

Position of the amide I' band at 1622 or 1625 cm^{-1} is the fundamental spectral difference between insulin amyloids

grown spontaneously (unseeded) at 60°C in the presence or absence of 20% (v/v) d_1 -deuterated ethanol (EtOD), respectively (Fig. 2A). Although in either case, the band's shape and position are characteristic for the amyloid's parallel β -sheet structure, it is red-shifted for EtOD-grown samples (1622 versus 1625 cm^{-1} for D_2O -grown amyloid; Fig. 2A). According to corresponding second derivative spectra (Fig. 2B), this shift should be specifically attributed to an increasing β -sheet fraction at 1620 cm^{-1} . Final, mature amyloid fibrils do not exhibit any spectral changes after being re-suspended in an alternative solvent (data not shown).

Insulin amyloid was seeded by mixing a small amount (5% (w/w) of the total protein content) of sonicated (Saborio et al. 2001) fibrils of either preparation with fresh insulin solutions (either with or without 20% (v/v) EtOD). Should the insulin solution be the same as the one originally used for spontaneous growth of the seeds, this was "homogenous seeding," as opposed to the "heterogeneous seeding" with alternative amyloid. The infrared spectra of each amyloid obtained through the homogenous seeding are very similar to the spectra of the spontaneously formed precursor seeds (Fig. 2). Minor differences are visible $\sim 1660\text{ cm}^{-1}$, namely, in the spectral region assigned to turns. Interestingly, templates of each type induce practically identical amyloid (in terms of its spectral features), when used for heterogeneous seeding (Fig. 2). This means that the critical factor, which determines spectral characteristics of growing amyloid is the preformed template, rather than the solvent conditions.

The FT-IR data were subsequently used for plotting time-dependencies of the amide I' band position (Fig. 3A), its bandwidth at half height (Fig. 3B), and a relative change of the β -sheet content (Fig. 3C). The plots show that the spectral changes occur immediately after doping the native insulin with the amyloid. The fastest conformational transi-

Figure 1. Seeds-induced aggregation of insulin. (A) Time-dependent changes in FT-IR spectra show the gradual evolution of the amide I' band of insulin reflecting the structural transition from the native form to amyloid: 2% (w/w) bovine insulin in 0.1 M NaCl in D_2O , pD = 1.9, seeded at 25°C at 20:1 weight ratio with preformed seeds. The total lapse time between the first and the final spectrum was 20 h. Arrows show directions of the spectral changes. (B) A height-scan AFM image of the final insulin aggregate.

Figure 2. Type of the original template determines infrared spectral features of the grown amyloid. The amide I' infrared band of insulin amyloids seeded homogeneously and heterogeneously. The original absorption (A) and the corresponding Savitzky-Golay second derivative (B) FT-IR spectra (solvent/seed): D₂O, 0.1 M NaCl in D₂O (pD 1.9); EtOD, 20% (v/v) EtOD in 0.1 M NaCl in D₂O (pD 1.9). Overlaid are spectra of insulin amyloid formed spontaneously at 60°C in EtOD (dashed line) or D₂O (dotted line).

tion takes place in the homogeneously seeded ethanol-containing insulin solutions. Apparently, each type of seeds acts as a more effective catalysts in the insulin solution of its original growth. Figure 3, A and B, shows that both the peak position and bandwidth of the final amyloid depend on the seed, rather than the cosolvent. On the other hand, the presence of ethanol leads to a more pronounced (by roughly 2 to 3 cm⁻¹) broadening of the amide I' band at an intermediate stage of the aggregation process (Fig. 3B). The relative spectral intensity at 1625/1622 cm⁻¹ quantifies the actual amount of the aggregated β -strands (Fig. 3C). The plots calculated for the homogeneously seeded amyloids show a linear versus time accumulation of the aggregated protein, and the other two corresponding to the heterogeneously seeded amyloids display some curvature. Especially, seeding insulin dissolved in 20% (v/v) EtOD with D₂O-grown amyloids evokes a sigmoid-like shape of the corresponding curve in Figure 3C.

Discussion

A red shift of the amide I' band in a hydrogen-bonded system, such as the insulin amyloid growing spontaneously in 20% (v/v) EtOD, is interpreted as strengthening of inter-chain hydrogen bonding (Nielsen et al. 2001). The second derivative spectra reveal that the EtOD type of amyloid has a higher fraction of the strongly hydrogen-bonded component of the β -sheet at 1620 cm⁻¹. The different energy of the inter- β -sheet hydrogen bonding is likely to reflect subtle structural differences between the two types of fiber. The fact that the spectra of once-formed amyloid remain intact

after resuspending in another solvent demonstrates that its fine, spectrally distinguishable structural features are well conserved within the fiber. Furthermore, as protein aggregation is said to drive the protein molecules to the "real" global energy minimum, the fact that slow spontaneous aggregation results in particularly tuned β -sheet conformation suggests that, under given solvent conditions, this structure corresponds to such an energy minimum (Gazit 2002). The experiments with homogenous seeding have showed rather unsurprisingly that, under conditions favoring particular amyloid structure and in the presence of seeds spontaneously formed under similar conditions, the growing amyloid will only maintain the fold of its initial template. Compared with the seeded aggregation at 25°C, the higher temperature (60°C) required for the spontaneous growth of amyloid appears to promote turns. Thus, it seems reasonable to attribute the presence of such less-ordered structural components to increased thermal fluctuations of polypeptide chains, which are expected to hamper ideal packing of the insulin molecules within the fibrils. Interestingly, the heterogeneous seeding experiments concluded in Figure 2 prove that slightly altered structure of the foreign seed is capable of "hijacking" the protein conformation by pushing it off the preferential aggregation pathway.

Recent studies have produced evidence that the "strains" reflect multiple distinct conformations of the prion (e.g., human [Wadsworth et al. 1999], hamster [Peretz et al. 2002], yeast [Chien and Weissman 2001; Chien et al. 2003]). Both the insulin amyloid types obtained from the single protein propagate, maintaining the original infrared features even under conditions favoring a different fold in

Figure 3. Kinetics of the homogeneously and heterogeneously seeded aggregation of insulin at 25°C followed as maximum of the amide I' band (A), the amide I' band's width at the half-height (HHBW; B), and relative absorption of the spectral component assigned to the β -sheet (C; solvent/seed): EtOD/EtOD (circles), D₂O/D₂O (squares), EtOD/D₂O (triangles), and D₂O/EtOD (diamonds).

the β -sheet fibers (Fig. 3A,B). In light of these facts, the two distinct conformations of insulin amyloid appear to have "strain"-like character.

That a foreign insulin seed still acts as an efficient catalyst of fibrillation reminds of the *in vitro* "promiscuity" reported for a chimera of the Sup35 yeast prion protein, which was effectively seeded by homogenous and heterogeneous amyloid templates (Chien and Weissman 2001; Chien et al. 2003). Similar degree of "tolerance" of the native protein toward different amyloid templates was observed for human lysozyme cross-seeded with its mutant fibrils (Morozova-Roche et al. 2000). However, a homologous seed does not always act as a more effective catalyst of the conformational transition, as is convincingly illustrated by the case of water-dissolved insulin seeded with amyloids grown in presence and absence of EtOD (Fig. 3).

When comparing kinetics of seed-induced aggregation of insulin in D₂O, and in 20% (v/v) EtOD, one must take into account that insulin dimers predominate in the former case

(Whittingham et al. 2002), and the presence of ethanol promotes monomers (Millican and Brems 1994). The water-grown seeds are more effective in insulin solutions containing dimers than in the presence of monomer-inducing ethanol, which sheds light on a possible mechanism of the aggregation. Namely, although (under different conditions) human insulin has been shown to aggregate through a monomeric intermediate (Ahmad et al. 2003), the cross-seeding kinetic data presented in Figure 3 would easily fit to an aggregation scenario involving two parallel pathways: through a monomer (preferential pathway in 20% [v/v] ethanol) or through a dimer (preferential pathway in water). A similar case of a protein capable of aggregating through a dimeric intermediate is transthyretin (Serag et al. 2001). Nevertheless, more subtle than "oligomeric competence," factors such as structural fluctuations, may play a role in the controlling kinetics of amyloidogenesis. The relative intensity of the β -sheet spectral component plotted as a function of time quantifies the progress of the aggregation.

The deviation from linearity observed for the heterogeneously seeded amyloids is puzzling. The sigmoidal shape of the plot corresponding to the aggregation of insulin in the presence of ethanol initiated with water-grown amyloid reflects increasing rate of the structural conversion. It could be hypothesized that such an enhancing effect stems from newly formed second-generation amyloid gradually acquiring higher affinity to the abundant monomeric substrate, therefore becoming a more effective aggregation catalyst in the EtOD solutions. The kinetic obstacles revealed in the heterogeneous seeding experiments remind one of barriers facing prion cross-species propagation (Chien and Weissman 2001; Peretz et al. 2002; Chien et al. 2003). On the other hand, the time-dependent enhancement of the catalytic properties of foreign fibrils appears to parallel the observation that heterogeneously seeded second generation of AApoAII(B) protein fibrils grown *in vivo* induced faster and more severe disease in mice (Xing et al. 2002). In other words, the sigmoidal shape of α/β -transition curve in EtOD-solv./D₂O-seed aggregation is likely to mirror refining catalytic properties of the growing amyloid. Despite these changes, the final FT-IR spectra continue to reflect the initial template (Fig. 2).

Figure 3B depicts time-dependencies of the amide I' bandwidth, which is a yardstick of structural fluctuations of a protein molecule. Increasing conformational fluctuations, which lead to disorder and unfolding of a protein, result in a marked broadening of the amide I' band (Dzwolak et al. 2003). By the same token, a reversed process of protein folding and subsequent assembling of native monomers into a compact and stable quaternary structure is expected to reduce, or simply damp, the fluctuations, leading to narrowing of the band. This has been confirmed in a very recent Raman spectroscopic study, which specifically coupled narrowing of the amide I band to the damping of conformational fluctuations in insulin molecules, upon their assembly into higher oligomers, as well as a result of amyloid formation (Dong et al. 2003). Figure 3B shows that the aggregation initially causes broadening of the amide I' band up to 57 cm⁻¹, which should be attributed to spectral separation between the transiently coexisting the native α -helices and β -sheet. However, it seems quite likely that partly unfolded aggregation-intermediate states contribute to the broadening of the spectral band, as well (Ahmad et al. 2003). The EtOD-grown seeds render the final half height bandwidth (HHBW) larger by ~5 cm⁻¹, suggesting a sustained degree of conformational fluctuations in the fibrils.

We have shown that two distinct amyloid states may be induced *in vitro* in a single, genetically unrelated to prions, protein-bovine insulin. The both amyloid states replicate maintaining their structure-hallmarking infrared features. The cross-seeding specificity and the kinetic features of both the amyloid types appear to mirror certain aspects of the prion strains phenomenon and kinetic traits observed in

other amyloidosis. This suggests that the strain-dependent infectivity of prions is an inherent feature of protein amyloids and, as such, remains in accordance with the protein-only molecular basis of prion diseases.

Materials and methods

Samples

Bovine pancreatic insulin was purchased from Sigma. D₂O, DCl, and ethanol were bought from Aldrich, Germany. For seeding experiments, 2% (w/w) solution of insulin in 0.1 M NaCl and D₂O (D₂O samples), or in 20% (v/v) EtOD, 0.1 M NaCl, and D₂O (EtOD samples) were pH-adjusted to 1.9 (pH-meter readout, +0.4; Makhatadze et al. 1995). Seeds were obtained under identical solvent conditions through unseeded spontaneous aggregation at 60°C. Prior to mixing with fresh insulin solutions (at 1:20 protein/weight ratio), the seeds were subjected to 30-min sonification. Immediately after the mixing, the samples were transferred to a thermostated FTIR cell by a flow-through system. Vanishing of the amide II band at 1550 cm⁻¹ confirmed that the final insulin amyloid was fully deuterated.

FT-IR spectroscopy

For FTIR measurements, CaF₂ transmission windows and 0.05 mm Teflon spacers were used. Temperature in the cell was controlled through an external water-circuit. All the FTIR spectra were collected on a Nicolet NEXUS FT-IR spectrometer equipped with a liquid nitrogen-cooled MTC detector. For each spectrum, 256 interferograms of 2 cm⁻¹ resolution were co added. The sample chamber was continuously purged with CO₂-free dry air. From the spectrum of each sample, a corresponding buffer spectrum was subtracted. All the spectra were baseline-corrected and normalized prior to further data processing. The semiquantitative plots of the progress of α -helix-to- β -sheet refolding upon aggregation in Figure 3C were calculated as $(I - I_\alpha)/(I_\beta - I_\alpha)$, where I_α is spectral intensity at 1625 (or 1622 cm⁻¹ in EtOD) of the native insulin (corresponding to the first spectrum), I_β is the intensity after complete aggregation, and I is a transient intensity at this wavenumber. All data processing was performed with GRAMS software (ThermoNicolet).

Atomic force microscopy

Samples for AFM imaging were diluted with water to the final concentration of 2.5 mg/mL. Thirty milliliters of the diluted amyloid sample was applied onto freshly cleaved mica (muscovite mica from Plano GmbH). After drying the samples in air (1–2 h), the data were acquired in tapping mode on a MultiModeTM SPM AFM microscope equipped with a Nanoscope IIIa Controller from Digital Instruments. In the AFM probes, Silicon SPM Sensors "NCHR" (force constant, 42 N/m; length, 125 mm; resonance frequency, 300 kHz) from Nanosensors were used.

Acknowledgments

This work was supported in part by the European Commission, grant no. G1MA-CT-2002-04055 (PRENABIO). V.S.'s participation was enabled by a European Commission-sponsored scholar-

ship grant "Support for Centers of Excellence" no. ICA1-CT-2000-70005.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Ahmad, A., Millett, I.S., Doniach, S., Uversky, V.N., and Fink, A.L. 2003. Partially folded intermediates in insulin fibrillation. *Biochemistry* **42**: 11404–11416.
- Bouchard, M., Zurdo, J., Nettleton, E.J., Dobson, C.M., and Robinson, C.V. 2000. Formation of insulin amyloid fibrils followed by FTIR simultaneously with CD and electron microscopy. *Protein Sci.* **9**: 1960–1967.
- Caughey, B., Raymond, G.J., and Bessen, R.A. 1998. Strain-dependent differences in β -sheet conformations of abnormal prion protein. *J. Biol. Chem.* **273**: 32230–32235.
- Chien, P. and Weissman, J.S. 2001. Conformational diversity in a yeast prion dictates its seeding specificity. *Nature* **410**: 223–227.
- Chien, P., DePace, A.H., Collins, S.R., and Weissman, J.S. 2003. Generation of prion transmission barriers by mutational control of amyloid conformations. *Nature* **424**: 948–951.
- Cohen, F.E. and Kelly, J.W. 2003. Therapeutic approaches to protein-misfolding diseases. *Nature* **426**: 905–909.
- Dong, J., Wan, Z.L., Popov, M., Carey, P.R., and Weiss, M.A. 2003. Insulin assembly damps conformational fluctuations: Raman analysis of amide I linewidths in native states and fibrils. *J. Mol. Biol.* **330**: 431–442.
- Dzwolak, W., Kato, M., Shimizu, A., and Taniguchi, Y. 2002. Fourier transform infrared spectroscopy in high-pressure studies on proteins. *Biochim. Biophys. Acta* **1595**: 131–144.
- Dzwolak, W., Ravindra, R., Lendermann, J., and Winter, R. 2003. Aggregation of bovine insulin probed by DSC/PPC calorimetry and FTIR spectroscopy. *Biochemistry* **42**: 11347–11355.
- Fabian, H., Choo, L.P., Szendrei, G.I., Jackson, M., Halliday, W.C., Otvos, L., and Mantsch, H.H. 1993. Infrared spectroscopic characterization of Alzheimer plaques. *Appl. Spectrosc.* **47**: 1513–1518.
- Fandrich, M. and Dobson, C.M. 2002. The behaviour of polyamino acids reveals an inverse side chain effect in amyloid structure formation. *EMBO J.* **21**: 5682–5690.
- Gazit, E. 2002. The "correctly folded" state of proteins: Is it a metastable state? *Angew. Chem. Int. Ed.* **41**: 257–259.
- Makhatazde, G.I., Clore, G.M., and Gronenborn, A.M. 1995. Solvent isotope effect and protein stability. *Nat. Struct. Biol.* **2**: 852–855.
- Millican, R.L. and Brems, D.N. 1994. Equilibrium intermediates in the denaturation of human insulin and two monomeric insulin analogs. *Biochemistry* **33**: 1116–1124.
- Morozova-Roche, L.A., Zurdo, J., Spencer, A., Noppe, W., Receveur, V., Archer, D.B., Joniau, M., and Dobson, C.M. 2000. Amyloid fibril formation and seeding by wild-type human lysozyme and its disease-related mutational variants. *J. Struct. Biol.* **130**: 339–351.
- Nielsen, L., Frokjaer, S., Carpenter, J.F., and Brange, J. 2001. Studies of the structure of insulin fibrils by Fourier transform infrared (FTIR) spectroscopy and electron microscopy. *J. Pharm. Sci.* **90**: 29–37.
- Peretz, D., Williamson, R.A., Legname, G., Matsunaga, Y., Vergara, J., Burton, D.R., DeArmond, S.J., Prusiner, S.B., and Scott, M.R. 2002. A change in the conformation of prions accompanies the emergence of a new prion strain. *Neuron* **34**: 921–932.
- Saborio, G.P., Permann, B., and Soto, C. 2001. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. *Nature* **411**: 810–813.
- Serag, A.A., Altenbach, C., Gingery, M., Hubbell, W.L., and Yeates, T.O. 2001. Identification of a subunit interface in transthyretin amyloid fibrils: Evidence for self-assembly from oligomeric building blocks. *Biochemistry* **40**: 9089–9096.
- Wadsworth, J.D.F., Hill, A.F., Joiner, S., Jackson, G.S., Clarke, A.R., and Collinge, J. 1999. Strain-specific prion–protein conformation determined by metal ions. *Nat. Cell Biol.* **1**: 55–59.
- Whittingham, J.L., Scott, D.J., Chance, K., Wilson, A., Finch, J., Brange, J., and Dodson, G.G. 2002. Insulin at pH 2: Structural analysis of the conditions promoting insulin fibre formation. *J. Mol. Biol.* **318**: 479–490.
- Xing, Y.M., Nakamura, A., Korenaga, T., Guo, Z.J., Yao, J.J., Fu, X.Y., Matsushita, T., Kogishi, K., Hosokawa, M., Kametani, F., et al. 2002. Induction of protein conformational change in mouse senile amyloidosis. *J. Biol. Chem.* **277**: 33164–33169.