Polymer Journal, Vol. 38, No. 3, pp. 302–305 (2006)

SHORT COMMUNICATIONS

Pre-incubation under High Pressure Accelerates Amyloid Formation from Insulin

Yasunori FUJIMOTO, Naoki TANAKA, and Shigeru KUNUGI†

Department of Polymer Science and Engineering, Kyoto Institute of Technology, Matsugasaki-Hashigamichou 1, Sakyo-ku, Kyoto 606-8585, Japan

(Received September 2, 2005; Accepted October 25, 2005; Published March 15, 2006)

KEY WORDS Insulin / Amyloid / High Pressure / Protein / Denaturation / Aggregation / [DOI 10.1295/polymj.38.302]

Recently it has been made clear that some associations or aggregations of abnormally folded proteins, such as β-amyloids, are stably present even under physiological condition, and those structures might cause specific diseases called conformation diseases; amyloidosis.[1–5] The present understandings are that a variety of proteins can form amyloids, fibrous aggregations of protein β-forms, besides some specific proteins concerned to the diseases.[6–10] Basic aspects of amyloid-formation mechanism have been studied by using many types of proteins and various physical and chemical methodologies.[11–17] Insulin is one of the prototype agents for such fundamental studies[18–24] and, as a physical perturbation, pressure (high hydrostatic pressure), besides temperature, is utilized in order to control the structural changes.[25–32]

In aqueous solutions, insulin exists as a mixture of monomer, dimer and hexamer; mainly in hexamer at pH around 7, in dimer at pH 2.0, and in monomer in 20% acetic acid.[32–34] Above room temperature, insulin can form amyloids and the amyloid is performed by nucleation-dependent elongation model. In this model the whole process is divided into three steps; nucleation, elongation and equilibrium and the rate determining step is considered as in the nucleation.[20,21,24] Dissociation to monomers from insulin dimers or hexamers and the following conformational change into modified monomers have to occur in prior to the nucleus formation. Even after amyloid is formed, three disulphide bonds in insulin are present intact without scissions.[35]

Here we have investigated some fundamental but important points of high pressure effects on β-amyloid formation from insulin.

EXPERIMENTAL

The amyloid formation process was observed by adding fluorescent probe, thioflavine T (ThT: Basic Yellow 1) 20 mM, in the insulin solutions. ThT shows a strong and characteristic fluorescence (ex. 450 nm and em. 480–490 nm), when it is captured in amyloid fibrils, although the details of the mechanisms are not yet fully elucidated.[36,37]

Bovine insulin and ThT were purchased from Sigma (St. Louis, Mo. USA). Insulin was dissolved in deionized and filtered water, and pH was adjusted to 2 by adding dilute HCl aqueous solution. The insulin concentration was determined spectrophotometrically ($A^{1\%}_{276\,nm} = 1$)[38] by using Shimadzu spectrophotometer UV-2200. The sample solution containing 5–20 mg/mL of insulin and 20 mM of ThT was portioned and set in a high-pressure cell settled in a fluorescence photometer (Shimadzu RF 5300). The details of the high-pressure equipment have been given elsewhere.[39] The excitation and emission wavelengths were set at 444 and 485 nm, respectively. After fluorescence measurements, the sample solutions were taken out and stained with 3% tungsto(VI) phosphoric acid aqueous solution for electron microscopic observation by JEOL-1220 (80 kV) (Tokyo, Japan).

RESULTS AND DISCUSSION

The time courses of fluorescence change of the insulin solution, after the temperature was raised at 60 °C, are typically as shown in Figure 1. As has been reported,[20,21,24] sigmoidal curves were obtained. They consist of the first gentle lag phase, greatly increasing growth phase, and the last gentle equilibrium phase. The reaction profiles can be phenomenologically ana-

†To whom correspondence should be addressed (Tel: +81-75-724-7003, Fax: +81-75-724-7100, E-mail: kunugi@kit.ac.jp).

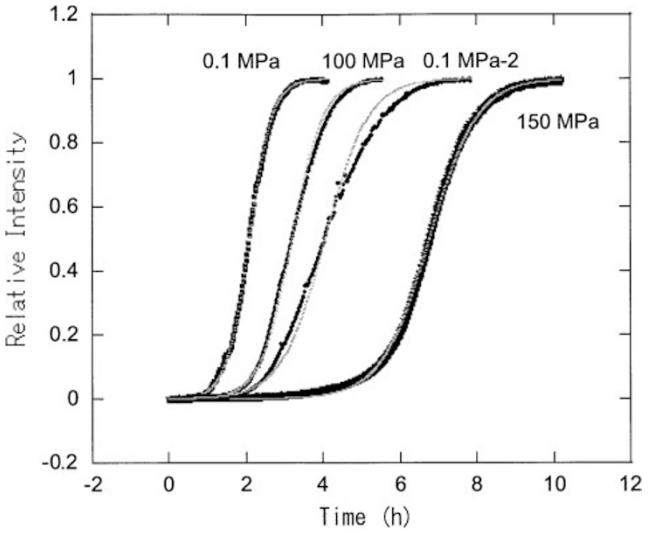

Figure 1. Time course of the fluorescence change from insulin solution containing 20 mM ThT at 60 °C. Pressures are indicated at the curves. The reaction time was counted after the temperature of the solution was increased to 60 °C. [insulin] = 10 mg/mL except for 0.1 MPa-2 (5 mg/mL). Gray curves are indicating the prediction of the change by using the parameters shown in Table I and eq 1 in the text.

lyzed by the following equation.[20]

$$F(t) = (F_i + m_i{}^*t) + (F_f + m_f{}^*t) /(1 + \exp(-(t - t_{1/2})/\tau)), \quad (1)$$

where $F(t)$ is the fluorescence intensity at time t, F_i and F_f are the initial and final fluorescence intensities, m_i and m_f are the inclination coefficients of the initial and final linear portions, and $t_{1/2}$ is the time to 50% of maximal fluorescence. For our experimental results, m_i and m_f can be considered as zero, and then eq 1 is simplified as;

$$F(t) - y_i = F'(t) = F_f/(1 + \exp(-(t - t_{1/2})/\tau)). \quad (2)$$

By plotting $\ln[\{F_f/F'(t)\}^{-1}]$ *vs.* t, $-1/\tau$ was obtained as the slope.

In this analysis, the lag-time (t_{lag}) and the extension rate coefficient (k_{ex}) were defined as, $t_{lag} = t_{1/2} - 2\tau$ and $k_{ex} = 1/\tau$. By the least square fitting of the whole profile, t_{lag} and k_{ex} were evaluated, and the insulin concentration and pressure dependence of these parameters are as listed in Table I.

Dissociation to monomers, structural change leading to modified monomer, and nucleation from the latter, all of these are considered to be reflected in the parameter t_{lag}. Thereafter, amyloid extension process starts from the nucleus, which is mainly evaluated through the parameter k_{ex}. The parameter t_{lag} decreased with increase in insulin concentration. k_{ex} also increased with the increase in insulin concentration.

High pressure suppressed the amyloid formation of insulin. With increasing pressure, t_{lag} increased drastically and, at the same time, k_{ex} decreased. Hence, at 400 or 300 MPa, we observed no fluorescence change even after 24 h (data not shown). Both nucleation and extension were slowed down by giving high pressure. Jansen *et al.* reported that specific circularly shaped insulin amyloids were obtained, by incubating under elevated pressures,[40] but only normal fibril formations (photographs not shown) were observed by electron microscopy. It is generally known that pressure can prevent the aggregation of proteins, especially of deformed (denatured) proteins. The main mechanism of pressure retardation of amyloid formation might be the suppression of both nuclear formation and further aggregation of modified monomer to extend the amyloid fibrils.

Table I. k_{ex} and t_{lag} parameters evaluated from ThT fluorescence change profiles of insulin solution under various conditions[a]

Insulin Conc. (mg/mL)	Pressure (MPa)	k_{ex} (h^{-1})	t_0 (h)	t_{lag} (h)
5	0.1	1.8	4.0	2.9
10	0.1	3.5	2.1	1.5
20	0.1	4.2	1.5	1.1
10	100	2.4	3.2	2.4
10	150	1.6	6.7	5.5
10	200	1.0	12.9	11.0
5	100 → 0.1[b]	3.8	3.1	2.0
5	200 → 0.1[b]	> 10	< 1.7	*ca.* 1.5

[a]at pH 2 and 60 °C. [b]Measured at atmospheric pressure after high-pressure pre-incubation for 1 h.

Although the amyloid formation of insulin was suppressed by high pressure, the application of high pressure on the once formed amyloids suspensions did not effectively resolve them (data not shown). On the other hand, drastic accelerations were observed by pre-incubating insulin solutions under pressurized conditions (after the applied pressure was released). As shown in Figure 2, exposure to the ambient pressure after 1 h incubation under 200 MPa, the ThT fluorescence increased very rapidly. The nucleation mechanism seems to be still effective but both nucleation and extension were accelerated, as known from the regressed parameters for those 100 MPa pre-incubation (Table I). These results can be explained by assuming that the dissociation of multimer forms of insulin into monomer and also the change of monomer into modified monomer or pre-amyloid structures are thermodynamically promoted under high pressure. For a number of proteins, it has been reported that the higher order associations of subunits tend to dissociate,[41–43] and partly deformed monomer proteins are stabilized,[44–46] under elevated pressures. In the case of pre-incubation at 200 MPa, the curve-fitting was not precisely performed, since the observed fluorescence

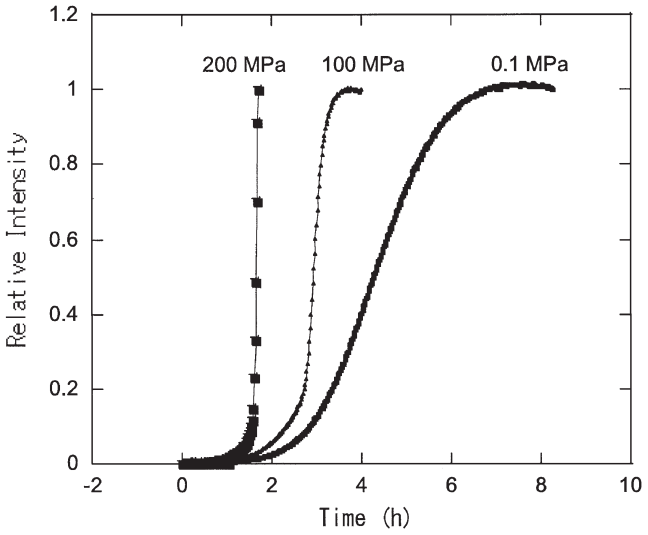

Figure 2. Time course of the fluorescence change from insulin solution containing 20 mM ThT at 60 °C after 1 h pre-incubation at the indicated pressure. Note that the changes were observed at 0.1 MPa after the release of pressure.

intensity exceeded the detecting limit of the fluorescence photometer. These results show that the mechanism of the association of the fluorescent probes and the fibrils was also modified by changing the pre-incubating conditions. The present results indicate that special cautions should be taken in the processes after the pressure release (thermal quenching, quick sample handling etc.), when the effects of pressure on the aggregation of this kind of proteins are studied by *ex citu* methodology.

REFERENCES

1. B. S. Prusiner, *Proc. Natl. Acad. Sci. U. S. A.*, **95**, 13363 (1998).
2. T. P. Lansbury, *Proc. Natl. Acad. Sci. U. S. A.*, **96**, 3342 (1999).
3. E. H. Koo, P. T. Lansbury, Jr., and J. W. Kelly, *Proc. Natl. Acad. Sci. U. S. A.*, **96**, 9989 (1999).
4. N. Sinha, C. J. Tsai, and R. Nussinov, *Protein Eng.*, **14**, 93 (2001).
5. M. C. Dobson, *Nature*, **426**, 884 (2003).
6. S. Goda, K. Takano, Y. Yamagata, R. Nagata, H. Akutsu, S. Maki, K. Namba, and K. Yutani, *Protein Sci.*, **9**, 369 (2000).
7. G. Damaschun, H. Damaschun, H. Fabian, K. Gast, R. Krober, M. Wieske, and D. Zirwer, *Proteins*, **39**, 204 (2000).
8. T. Konno, *Protein Sci.*, **10**, 2093 (2001).
9. P. O. Souillac, V. N. Uversky, I. S. Millett, R. Khurana, S. Doniach, and A. L. Fink, *J. Biol. Chem.*, **277**, 12657 (2002).
10. A. J. Modler, K. Gast, G. Lutsch, and G. Damaschun, *J. Mol. Biol.*, **325**, 135 (2003).
11. Y. Goto, N. Takahashi, and A. L. Fink, *Biochemistry*, **29**, 3480 (1990).
12. G. J. Miroy, Z. Lai, H. A. Lashuel, S. A. Peterson, C. Strang, and J. W. Kelly, *Proc. Natl. Acad. Sci. U. S. A.*, **93**, 15051 (1996).
13. Y. C. Kudva, H. J. Hiddinga, P. C. Butler, C. S. Mueske, and N. L. Eberhardt, *FEBS Lett.*, **416**, 117 (1997).
14. V. N. Uversky, G. Yamin, P. O. Souillac, J. Goers, C. B. Glaser, and A. L. Fink, *FEBS Lett.*, **517**, 239 (2002).
15. W. Dzwolak, R. Ravindra, J. Lendermann, and R. Winter, *Biochemistry*, **42**, 11347 (2003).
16. L. A. Munishkina, J. Henriques, V. N. Uversky, and A. L. Fink, *Biochemistry*, **43**, 3289 (2004).
17. A. Arora, C. Ha, and C. B. Park, *FEBS Lett.*, **564**, 121 (2004).
18. V. Sluzky, J. A. Tamada, A. M. Klibanov, and R. Langer, *Proc. Natl. Acad. Sci. U. S. A.*, **88**, 9377 (1991).
19. M. Bouchard, J. Zurdo, E. J. Nettleton, C. M. Dobson, and C. V. Robinson, *Protein Sci.*, **9**, 1960 (2000).
20. L. Nielsen, R. Khurana, A. Coats, S. Frokjaer, J. Brange, S. Vyas, V. N. Uversky, and A. L. Fink, *Biochemistry*, **40**, 6036 (2001).
21. M. R. H. Krebs, E. H. C. Bromley, S. S. Rogers, and A. M. Donald, *Biophys. J.*, **88**, 2013 (2005).
22. A. Ahmad, I. S. Millett, S. Doniach, V. N. Uversky, and A. L. Fink, *Biochemistry*, **42**, 11404 (2003).
23. M. R. H. Krebs, C. E. MacPhee, A. F. Miller, I. E. Dunlop, C. M. Dobson, and A. M. Donald, *Proc. Natl. Acad. Sci. U. S. A.*, **101**, 14420 (2004).
24. D. Hamada and C. M. Dobson, *Protein Sci.*, **11**, 2417 (2002).
25. L. Smeller, P. Rubens, and K. Heremans, *Biochemistry*, **38**, 3816 (1999).
26. T. W. Randolph, M. Seefeldt, and J. F. Carpenter, *Biochem. Biophys. Acta*, **1595**, 224 (2002).
27. Y.-S. Kim, T. W. Randolph, F. J. Stevens, and J. F. Carpenter, *J. Biol. Chem.*, **277**, 27240 (2002).
28. J. Torrent, M. T. Alvarez-Martinez, F. Heitz, J.-P. Liautard, C. Balny, and R. Lange, *Biochemistry*, **42**, 1318 (2003).
29. F. G. De Felice, M. N. N. Vieira, M. Nazareth, L. Meirelles, L. A. Morozova-Roche, C. M. Dobson, and S. T. Ferreira, *FASEB J.*, **18**, 1099 (2004).
30. J. Torrent, M. T. Alvarez-Martinez, M. C. Harricane, F. Heitz, J. P. Liautard, C. Balny, and R. Lange, *Biochemistry*, **43**, 7162 (2004).
31. Y. O. Kamatari, S. Yokoyama, H. Tachibana, and K. Akasaka, *J. Mol. Biol.*, **349**, 916 (2005).
32. D. F. Waugh and M. L. Sackler, *J. Am. Chem. Soc.*, **75**, 2592 (1953).
33. M. A. Weissl, Q. X. Hua, C. S. Lynch, B. H. Frank, and S. E. Shoelson, *Biochemistry*, **30**, 7373 (1991).
34. C. Bryant, M. Strohl, L. K. Green, H. B. Long, L. A. Alter, A. H. Pekar, R. E. Chance, and D. N. Brems, *Biochemistry*, **31**, 5692 (1992).
35. J. L. Jiménez, E. J. Nettleton, M. Bouchard, C. V. Robinson, C. M. Dobson, and H. R. Saibil, *Proc. Natl. Acad. Sci. U. S. A.*, **99**, 9196 (2002).
36. R. Khurana, V. N. Uversky, L. Nielsen, and A. L. Fink, *J. Biol. Chem.*, **276**, 22715 (2001).
37. M. R. Krebs, E. H. Bromley, and A. M. Donald, *J. Struct. Biol.*, **149**, 30 (2005).
38. R. R. Porter, *Biochim. J.*, **53**, 320 (1953).
39. T. Tanaka, H. Ikeuchi, N. Tanaka, and S. Kunugi, *Cell. Mol.*

Biol., **50**, 317 (2004).
40. R. Jansen, S. Grudzielanek, W. Dzwolak, and R. Winter, *J. Mol. Biol.*, **338**, 203 (2004).
41. R. J. St. John, J. F. Carpenter, C. Balny, and T. W. Randolph, *J. Biol. Chem.*, **276**, 46856 (2001).
42. M. Panda, J. Ybarra, and P. M. Horowitz, *J. Biol. Chem.*, **276**, 6253 (2001).
43. D. Foguel, M. C. Suarez, A. D. Ferrão-Gonzales, T. C. R. Porto, L. Palmieri, C. M. Einsiedler, L. R. Andrade, H. A. Lashuel, P. T. Lansbury, J. W. Kelly, and J. L. Silva, *Proc. Natl. Acad. Sci. U. S. A.*, **100**, 9831 (2003).
44. S. M. Martins, A. Chapeaurouge, and S. T. Ferreira, *J. Biol. Chem.*, **278**, 50449 (2003).
45. A. D. Ferrão-Gonzales, S. O. Souto, J. L. Silva, and D. Foguel, *Proc. Natl. Acad. Sci. U. S. A.*, **97**, 6445 (2000).
46. C. Dirix, F. Meersman, C. E. MacPhee, C. M. Dobson, and K. Heremans, *J. Mol. Biol.*, **347**, 903 (2005).