East Eur. J. Phys. Vol.1 No.4 (2014) 96-99

PACS: 61.05.cf, 87.14.E-, 87.14.em, 87.15.bk, 87.19.xr.

SMALL ANGLE X-RAY SCATTERING STUDY OF INSULIN FIBRILS

M.V. Romanova[1], I.L. Maliyov[1], M.S. Girych[1], E.A. Vus[1], D.I. Svergun[2], Al. Kikhney[2], C. Jeffries[2]

[1]Department of Nuclear and Medical Physics, V.N. Karazin Kharkiv National University
4 Svobody Sq., Kharkov, 61022, Ukraine;
[2]European Molecular Biology Laboratory (EMBL)
Hamburg Outstation, Notkestrasse 85, D22603 Hamburg, Germany.
e-mail: romanova-mari@mail.ru
Received August 13, 2014

The small-angle X-ray scattering technique was employed to determine low-resolution 3D structure of insulin amyloid fibrils. This object is of particular interest since amyloid deposits of insulin causes insulin injection amyloidosis. Structural characterization of amyloid fibrils as a particular class of linear highly ordered protein aggregates is of utmost importance for deeper understanding of the molecular etiology of conformational diseases and development of effective therapeutic strategies. The small-angle X-ray scattering pattern analysis showed that the maximum dimension of the insulin fibril cross-section reaches 24±2.4 nm, while gyration radius of the cross-section is about 6 nm.
KEY WORDS: amyloid fibrils, insulin, small angle X-ray scattering, Thioflavin T

ВИВЧЕННЯ ФІБРИЛ ІНСУЛІНУ МЕТОДОМ МАЛОКУТОВОГО РЕНТГЕНІВСЬКОГО РОЗСІЯННЯ

М.В. Романова[1], І.Л. Малійов[1], М.С. Гірич[1], К.О. Вус[1], Д.І. Свергун[2], О. Кікхней[2], С. Джефріс[2]

[1]Харківський національний університет імені В.Н. Каразіна
пл. Свободи, 4, Харків 61022, Україна
[2]Європейська лабораторія молекулярної біології
Ноткештрассе 85, Гамбург D22603, Німеччина
Метод малокутового розсіяння був застосований для визначення 3D структури низької роздільної здатності амілоїдних фібрил інсуліну. Інтерес до даного об'єкту обумовлений здатністю амілоїдних депозитів інсуліну викликати інсулін-опосередкований ін'єкційний амілоїдоз. Дослідження структурної організації амілоїдних фібрил, особливого класу лінійних високовпорядкованих білкових агрегатів, виключно важливе для більш глибокого розуміння молекулярної етіології конформаційних хвороб та створення ефективних терапевтичних стратегій. Аналіз паттернів малокутового розсіяння показав, що максимальний розмір перезізу фібрил інсуліну сягає 24±2,4 нм, а радіус обертання перерізу складає близько 6 нм.
КЛЮЧОВІ СЛОВА: амілоїдні фібрили, інсулін, малокутове рентгенівське розсіяння, тіофлавін Т

ИЗУЧЕНИЕ ФИБРИЛЛ ИНСУЛИНА МЕТОДОМ МАЛОУГЛОВОГО РЕНТГЕНОВСКОГО РАССЕЯНИЯ

М.В. Романова[1], І.Л. Малийов[1], М.С. Гирич[1], Е.А. Вус[1], Д.І. Свергун[2], О. Кикхней[2], С. Джефрис[2]

[1]Харьковский национальный университет имени В.Н. Каразина
пл. Свободы, 4, Харьков 61022, Украина
[2]Европейская лаборатория молекулярной биологии
Ноткештрассе 85, Гамбург D22603, Германия
Метод малого углового рассеяния был применен для определения 3D структуры низкого разрешения амилоидных фибрилл инсулина. Интерес к данному объекту связан со способностью амилоидных отложений инсулина вызывать инсулин-опосредованный инъекционный амилоидоз. Исследование структурной организации амилоидных фибрилл, особого класса линейных высокоупорядоченных белковых агрегатов, исключительно важно для более глубокого понимания молекулярной этиологии конформационных болезней и создания эффективных терапевтических стратегий. Анализ паттернов малоуглового рассеяния показал, что максимальный размер сечения фибрилл инсулина достигает 24±2,4 нм, а радиус вращения сечения составляет около 6 нм.
КЛЮЧЕВЫЕ СЛОВА: амилоидные фибриллы, инсулин, малоугловое рентгеновское рассеяние, тиофлавин Т

One of the most interesting properties of polypeptide chain involves its ability to one-dimensional crystallization with the formation of highly ordered insoluble assemblies (amyloid fibrils). These specific linear aggregates are currently associated with a number of conformational disorders, such as Alzheimer's, Parkinson's, Huntington's diseases, type II diabetes, rheumatoid arthritis, spongiform encephalopathy, systemic amyloidosis, etc.[1–3]. The biophysicists operate with a modern definition of amyloid fibrils, in which twisted and unbranched fibers typically have a diameter of about 10 nm and highly variable lengths up to several microns[4– 6]. The protofilaments are characterized by a core cross β-sheet structure in which continuous β-sheets run along the fibril axis and are formed from β-strands stacked by hydrogen bonds in the direction perpendicular to the long axis of the fibril [7,9]. Such a β-sheet structure produces a typical high resolution X-ray patterns with meridional reflection at 0.47 nm corresponding to the main chain spacing and a broader equatorial reflection at 0.8–0.12 nm determined by the side chain spacing [6,10,11]. The structural similarity of amyloid fibrils and their common features, *viz*. yellow-green birefringence on the association

© Romanova1 M.V., Maliyov I.L., Girych M.S., Vus E.A., Svergun D.I., Kikhney Al., Jeffries C., 2014

with specific dye Congo red and intense fluorescence on the binding to most prominent amyloid marker Thioflavin T suggest a common mechanism of fibrillization process [7,12].

Small-angle X-ray scattering (SAXS) is a structural method providing information about low resolution shape of macromolecules in solution. This method enables researchers to investigate biological samples in a nearly physiological environment and to analyze structural changes of biomacromolecules in response to variations of external conditions in the absence of their crystals [13,14]. SAXS is applicable to native particles in solution and is particularly suitable for the study of less structured systems. Importantly, SAXS can be applied to macromolecules possessing inherent flexibility, that are difficult to crystallize and their high resolution structure cannot be determined by Macromolecular X-ray Crystallography (MX). SAXS is often used as a complimentary tool for high resolution techniques, such as MX and Nuclear Magnetic Resonance (NMR), in particular, the methods have been developed to generate probable configurations of missing fragments in the incomplete high-resolution structures based on the solution SAXS patterns from the full length proteins, and they were successfully employed in practice [15-17].

In a typical scattering experiment the dissolved macromolecules are exposed to a collimated X-ray (SAXS) or neutron (SANS) beam and the scattered intensity *I(s)* is recorded by the detector. The experiments on macromolecules in solutions involve separate measurements of the scattering from the solution and the solvent. For dilute solutions (concentrations in mM range) the particles are chaotically distributed leading to isotropic intensity depending only on the scattering angle 2θ between the incident and scattered beam. For monodisperse solutions, the intensity *I(s)* obtained after subtraction of the separately measured solvent scattering is proportional to the scattering from a single particle averaged over all orientations [18]. To characterize the space of the object and estimate overall sizes of the object, the $p(r)$ distance distribution function, corresponding to the distribution of distances between volume elements inside the particle is often used. *I(s)* is related to $p(r)$ through Fourier transformation:

$$p(r) = \frac{r^2}{2\pi^2}\int_0^\infty s^2 I(s)\frac{\sin sr}{sr}ds; \qquad I(s) = 4\pi\int_0^{Dmax} p(r)\frac{\sin sr}{sr}dr. \quad (1)$$

In principle, the distance distribution function *p(r)* contains the same information as the scattering intensity *I(s)*, but the real space representation is more intuitive and allows the immediate evaluation of the largest particle dimensions by visual assay of *p(r)*[19].

Polypeptide hormone insulin is the most studied protein which is known to form fibrils at physiological conditions and *in vitro,* this fact making it a convenient model for the fibrillation investigations. Despite the growing body of research in this field, the absolute molecular mechanism of the fibrillation process is remain to be unexplained and more in depth knowledge of the structure of insulin amyloid fibrils is essential for understanding the mechanism involved in the formation of intracellular deposits, which causes insulin injection amyloidosis disease.

The goal of the present study was to gain insight into the structure of insulin mature fibrils. In the first part of work we studied the growth of insulin fibrils by fluorescent method. The formation of fibrils was confirmed by Thioflavin T assay. For structure determination SAXS technique was used for different protein concentrations. The structure investigations of insulin fibrils could not only help to understand the insulin-related disorders, but may also give a hint for developing of anti-amyloidogenic agents in general.

MATERIAL AND METHODS

SAXS data processing and analysis

Synchrotron radiation X-ray scattering data were collected from four solute concentrations of the fibrillar insulin in the range 0.3 to 2.0 mg/ml in glycine buffer on the “P12” camera of the European Molecular Biology Laboratory (EMBL) on storage ring “PETRA III” at Deutches Elektronen Synchrotron (DESY), Hamburg, Germany). The data were collected using a photon counting Pilatus 2M detector at a sample-detector distance of 3.1 m and a wavelength λ = 0.124 nm, the range of momentum transfer $0.06 < s < 3.5$ nm^{-1} was covered ($s = 4\pi \sin\theta/\lambda$, where 2θ is the scattering angle). To monitor the radiation damage, 20 successive 0.05 seconds exposures of insulin solutions were compared. The data were normalized to the intensity of the transmitted beam and radially averaged; the scattering of the buffer was subtracted and the difference curves were scaled for protein concentration.

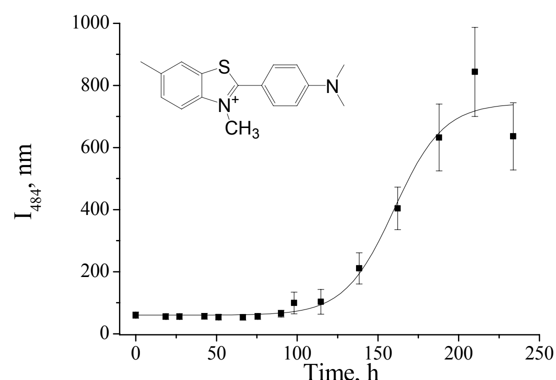

Fig. 1. Fluorescence intensity of Thioflavin T as a function of time. Shown in inset is the structural formula of ThT.

The data processing steps were performed using the program package “PRIMUS” [20]. The pair distribution function *p(r)* was computed using the indirect transform package “GNOM” [21].

Fibril formation and fluorescence studies

Bovine insulin (Ins) fibrillization was induced by continuous shaking of the proteins (10 mg/ml) for 10 days at 37 degrees, pH 1.6. The process of fibril formation was monitored by Thioflavin T (ThT) assay (Fig. 1). Fluorescence

measurements were performed with a LS-55 spectrofluorimeter equipped with a magnetically stirred cuvette holder (Perkin-Elmer Ltd., Beaconsfield, UK). The fluorescence intensity of amyloid insulin-bound ThT proved to be 12 times higher compared to the dye emission in the presence of native protein. Furthermore, the insulin amyloid formation was confirmed by the blue shift of ThT emission maximum by ca. 8 nm compared to the dye emission maximum in the presence of native protein.

RESULTS AND DISCUSSION

The processed SAXS pattern and distance distribution functions from insulin fibrils are displayed in Fig. 2,3. The nominal resolution of the scattering data is indicated as $d=2\pi/s$. Insulin mature fibrils may be micrometers long [6,22-24] whereas for our experimental range of momentum transfer ($0.06 < s < 3.5$ nm^{-1}) d_{max} is 100 nm, clearly, in this case only cross-sectional parameters are available and correspondingly less structural information can be obtained by SAXS.

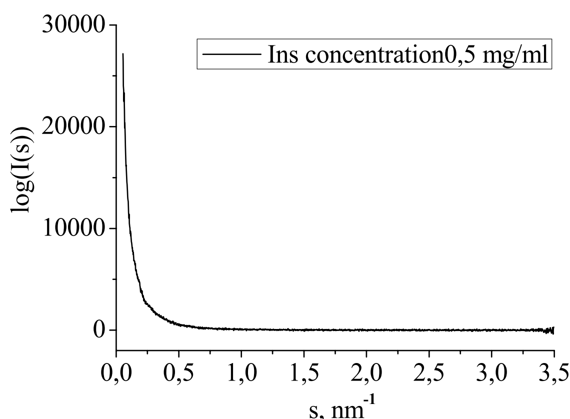

Fig.2 SAXS pattern of insulin fibrils.

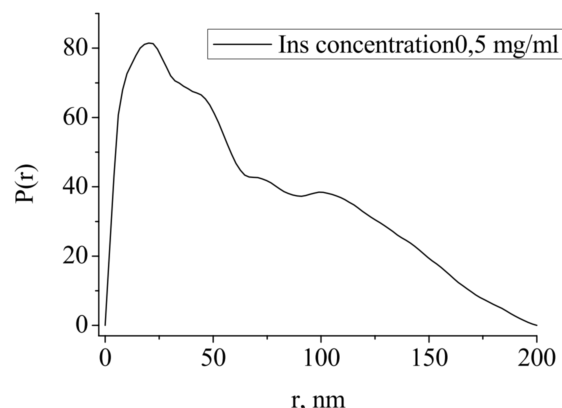

Fig.3. Distance distribution function for insulin fibrils.

Distance distribution function of cross-section is presented in Fig. 4. The scattering pattern analysis showed that the maximum dimension of the fibril cross-section is about 24 ± 2.4 nm, while the radius of gyration (Rg) of the cross-section is about 6 nm. The 100 nm peak in the p(r) could be interpreted as a repeating unit. One could say that cross-section can be represented as an ellipse with semiaxes 2 and 12 nm (i.e. 4 nm × 24 nm).

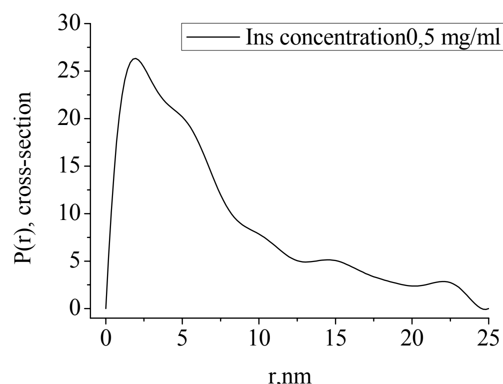

Fig.4. Distance distribution function of cross-section.

Recent Atomic Force Microscopy measurements of different fibrillar species of insulin showed mature fibrils to have a diameter of about 4 nm [25, 26]. According to the proposed model of fibril self-assembly, rodlike protofilaments interact with one another and form protofibrils, which typically consist of 2 or 3 intertwined protofilaments. Further interaction of protofibrils results in the assembly of mature fibrils. TEM studies of insulin fibrillization report fibril diameter ranging from 3 to 15 nm. In some cases, protofibrils (3 nm) are seen to bundle together in groups of 2–5 and yield mature fibrils that are 10–15 nm in diameter [27]. Based on our finding and the results reported elsewhere we assumed that insulin fibrils with a diameter 4 nm align to each other and form a ribbon like packing in the cross-section.

To summarize, SAXS data can be interpreted as follows: insulin fibrils have repeating units of 100 nm, presumably turn structure (e.g. consisting of two 50 nm motifs) with the prolate cross-section of dimension 4 nm × 24 nm, composed of six fibrils with 4nm diameter aligned together in a ribbon like manner.

REFERENCES

1. Dobson C.M. Protein folding and misfolding // Nature. – 2003. – Vol. 426. – P. 884–890.
2. Girych M.S., Maliyov I.L., Romanova M.V., et.al. Fluorescence energy transfer study into lipid bilayer interactions of truncated apolipoprotein a-i mutants // Biophys. – 2013. – Vol. 29(1). – P. 39-50.
3. Stefani M. Protein misfolding and aggregation: new examples in medicine and biology of the dark side of the protein world // Biochim. Biophys. Acta. – 2004. –Vol. 1739. – P. 5–25.
4. Greenwald J., Riek R. Biology of Amyloid: Structure, Function, and Regulation // Structure. – 2010. – Vol. 18. – P. 1244-1260.
5. Eichner T., Radford Sh. E. A Diversity Of Assembly Mechanisms Of A Generic Amyloid Fold // Molecular Cell. – 2011. – Vol. 43. – P. 8-18.
6. Vestergaard B., Groenning M., Roessle M., et.al. A Helical Structural Nucleus Is The Primary Elongating Unit Of Insulin Amyloid Fibrils // PLoS Biology. – 2007. –Vol.5. –P. 1089-1097.
7. Adamcik J., Mezzenga R. Study of amyloid fibrils via atomic force microscopy // Current Opinion in Colloid & Interface

Science. – 2012. – Vol. 17. – P. 369–376.
8. Jimenez J. L., Nettleton E. J., Bouchard M., et.al. The protofilament structure of insulin amyloid fibrils // PNAS. – 2002. – Vol. 99. – P. 9196–9201.
9. Yamamoto Sh., Watarai H. Raman Optical Activity Study on Insulin Amyloid and Prefibril Intermediate // Chirality. – 2012. – Vol. 24. – P. 97–103.
10. Berhanu W. M., Masunov A. E. Alternative Packing Modes Leading to Amyloid Polymorphism in Five Fragments Studied With Molecular Dynamics // PeptideScience. – 2011. –Vol. 98. – P. 131-144.
11. Greenwald J., Riek R. Biology of Amyloid: Structure, Function and Regulation // Structure. –2010. – Vol.18. – P. 1244-1260.
12. Swift B.. Examination of insulin injection sites: an unexpected finding of localized amyloidosis // Diabet. Med. – 2002. – Vol. 19. – P. 881–882.
13. Svergun D. Advanced solution scattering data analysis methods and their applications // J. Appl. Cryst. – 2000. – Vol. 33. – P. 530-534.
14. Svergun D. Mathematical methods in small-angle scattering data analysis // J. Appl. Cryst. – 2000. – Vol. 24. – P. 485-492.
15. Petoukhov M. V., Eady N. A., Brown K. A., Svergun, D. I. Addition of missing loops and domains to protein models by x-ray solution scattering. // Biophys. J. – 2002. –Vol. 83. –P. 3113-3125.
16. Garcia P. , Ucurum Z., Bucher R., et.al. Molecular insights into the self-assembly mechanism of dystrophia myotonica kinase // FASEB J. – 2006. –Vol. 20. –P. 1142-51.
17. Durand D., Cannella D., Dubosclard V., et.al. Small-angle X-ray scattering reveals an extended organization for the autoinhibitory resting state of the p47(phox) modular protein // Biochemistry. – 2006. – Vol. 45. – P. 7185-93.
18. Svergun D. I., Koch M.H.J. Small-angle scattering studies of biological macromolecules in solution // Rep. Prog. Phys. – 2003. – Vol. 66. – P. 1735–1782.
19. Glatter O., Kratky O. Small Angle X-ray Scattering. Ch.1 – London: Academic Press, 1982. – 7 p.
20. Konarev P.V., Volkov V.V., Sokolova A.V., et.al. PRIMUS – a Windows-PC based system for small-angle scattering data analysis // J. Appl. Cryst. – 2003. – Vol. 36. – P. 1277-1282.
21. Svergun D.I. Determination of the regularization parameter in indirect-transform methods using perceptual criteria // J. Appl. Crystallogr. – 1992. – Vol. 25. – P. 495-503.
22. Szymanska A., Hornowski T., Kozak M., Slosarek G. The SAXS and Rheological Studies of HEWL Amyloid Formation // Acta Physica Polonica. – 2008. – Vol. 114. – P. 447-454.
23. Kun Lu, Jacob J., Thiyagarajan P., et.al. Exploiting Amyloid Fibril Lamination for Nanotube Self-Assembly // J. AM. CHEM. SOC. – 2003. – Vol. 125. – P. 6391-6393.
24. Fitzpatrick A. W. P., Debelouchina G. T., Bayro M. J., et.al. Atomic structure and hierarchical assembly of a cross-β amyloid fibril // PNAS. – 2012. – Vol. 110. – P. 5468–5473.
25. Khurana R., Ionescu-Zanetti C., Pope M., et.al. A General Model for Amyloid Fibril Assembly Based on Morphological Studies Using Atomic Force Microscopy // Biophysical Journal. – 2003. – Vol. 85. – P. 1135–1144.
26. Jansen R., Dzwolak W., Winter R. Amyloidogenic Self-Assembly of Insulin Aggregates Probed by High Resolution Atomic Force Microscopy // Biophysical Journal. – 2005. – Vol. 88. – P. 1344–1353.
27. Brange J., Andersen L., Laursen E. D., et.al. Toward understanding insulin fibrillation // J. Pharm. Sci. – 1997. – Vol. 86. – P. 517–525.