Thermodynamic and structural studies of protein aggregation

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PHD THESIS

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Introduction

Our view of protein aggregation has been changed significantly in the last decades. Proteins are involved in almost every process of life, and they exhibit well-defined structure evolved for their function. All this is valid as long as the protein homeostasis is maintained. Proteins with non-native structure can accumulate in the body due to mutations, changes in the environment, or deficiency of the cellular quality control system. These processes cause the loss of function, and the alternative conformations are generally aggregation-prone.

Until recently, protein aggregation studies were focused on amyloid fibrils, which are long, fibrillar homopolymers. Amyloid deposits were observed in over 30 human and even more animal diseases including Alzheimer’s and Parkinson’s diseases and type-II diabetes.

It is well known that amyloid formation is a nucleation dependent process and small aggregates and oligomers are present in the early phase. However, the (patho)physiological effects of such early aggregates and oligomers have been studied only recently. In various diseases, a correlation between the presence of the oligomers and the symptoms has been shown. It is possible that the formation of amyloid fibrils is not a cause of the disease but a consequence where the toxic aggregate species form less harmful amyloid fibrils.

It has been discovered and studied since the early 2000s, that in many organisms (including humans), nontoxic, functional amyloids are involved in various cellular processes. For example, the amyloid form of the protein Pmel17 is essential for human melanin synthesis. Peptide hormones are stored as amyloids within endocrine secretory granules. Therefore, instead of being toxic, the amyloid form of some proteins are essential for the living cells.

The pharmaceutical industries develop and design increasingly efficient peptide and protein drugs to cure diseases. Upon administering, a high local concentration of the drug can be created, which implies the risk of aggregation. Injection-localized amyloidosis of insulin has been observed in diabetic patients. Another example of conformational diseases is dialyses-related amyloidosis developing as a side-effect of a medical treatment: long-term hemodialysis results amyloid deposits within the osteoarticular system.

In the light of these, it is important to gain information on the disease-related proteins. What is the structure (morphology and spatial structure) of the aggregates identified as expressing pathological effects? What are the biochemical processes leading to the formation of protein aggregates and how can we give their kinetic and thermodynamic characterization?
To investigate the aggregation mechanism and the structure of the aggregates, various model peptides and proteins, unrelated or related to any disease, are used. Most of the biochemical and biophysical techniques can be easily adapted to study protein aggregation.

In my PhD thesis, I made the effort to summarize the literature of the field; specify my aims; present and interpret the new scientific results. I prepared a longer introduction of CD spectroscopy for the better understanding. Moreover, I intended to provide a detailed help showing both the advantages and limitations in Hungarian for those who are interested in the technique of CD spectroscopy for the study of protein structure.

**Aims**

The main goal of our study was to investigate the thermodynamics and structural background of protein aggregation and development or improvement of the related methodology. This work focused on three topics on which we sought answers to the following questions:

1. Investigation of the thermal stability of β₂-microglobulin amyloid fibrils
   - Is the heat-denaturation of β₂-microglobulin amyloid fibrils a reversible process?
   - Is it possible to have the amyloid fibrils fully dissociated at high temperatures?
   - What is the repolymerization kinetics of the heat-treated samples?
   - Is the heat-treatment method applicable to the study the amyloid fibrils of other proteins?
   - Is it possible to characterize the effects of the environmental conditions by the heat-denaturation profile?

2. Aggregation of D9S(P) variant of Trp-cage miniprotein
   - What are the structural consequences of the side-chain modification?
   - What are the morphological and structural features of D9S(P) aggregates?
   - Is it possible to build a stable model of D9S(P) aggregates based on the triple helical structure of collagen?
   - Are the identified intermolecular interactions in the model experimentally verifiable?

3. Estimation of the secondary structure from protein CD spectra
• How to study amyloid fibrils by circular dichroism?
• Are the existing structure estimation methods usable to estimate the structure of aggregates?
• Is there any correlation between the orientation and twist of β-sheets and their CD spectra?
• Taking into account the effect of the twist, how can we develop an efficient algorithm to estimate protein secondary structure?
• Is it possible to predict the fold of a protein based on the information content provided by the secondary structure estimation?
• How does our method perform on protein aggregates and globular proteins?

Thesis points

1. The reversible heat-denaturation of β2-microglobulin amyloid fibrils involves two simultaneous processes: the dissociation of monomers from the fibril end and the breakage of fibrils.
   After 5-10 minutes incubation at 99°C, the amyloid fibrils formed at 37°C disaggregate into monomers. Upon subsequent incubation at 37°C, complete re-extension was observed. After the heat-treatment at 65-85°C for 5-10 min, the repolymerization rates were higher than the original growth rate at 37°C, indicating that the number of extendable fibril-ends increased due to the breakage of fibrils.

2. The effects of the growth condition on stability are reproducibly examined by the thermal denaturation profiles.
   The effects of high initial seed-concentration, the incubation time and temperature, and ions in the solution on thermal denaturation were reproducible in our experiments. Our method developed for studying the thermal stability can be generally used to characterize the stability of aggregates.

Figure 1. Heat-denaturation profiles of β2-microglobulin, K3-, and α-synuclein amyloid fibrils (left). Schematic representation of the possible thermal-induced disaggregation (breakage and depolymerization) of β2m amyloid fibrils (right).

3. D9S(P) variant of Trp-cage miniprotein form polyproline-II helical aggregates at 4°C.
D9S(P) forms fibrils showing a collagen-like polyproline-II-helix type CD spectrum with a negligible thioflavin-T binding capacity. Transmission electron microscopy images displayed long, thin and flexible fibrils, which differ from amyloid fibrils. Taking into account that the C-terminal half of the protein is rich in proline residues, these results suggest that D9S(P) forms collagen-like polyproline-II helix structure. We have built a PPII triple helical polymer model of the peptide. Intermolecular 16Arg-9Ser(P) salt-bridges arose upon the energy minimization, which can be validated experimentally. These results suggest that the phosphorylation of Ser9 residue destabilizes the native structure of monomers and initiates and stabilizes the formation of PP-II helical aggregates at 4°C.

Figure 2. Polyproline II helical model of D9S(P) filaments.

4. The orientation and twisting of β-sheets has a strong influence on the CD spectrum. Circular dichroism (CD) spectroscopy is a widely used technique for the study of protein structure. However, previous algorithms provide limited usability for the analysis of β-structure-rich proteins and protein aggregates. We showed that the observed spectral diversity of such proteins can be explained by the parallel-antiparallel orientation and the twist of the β-sheets.

5. The performance of secondary structure prediction of proteins from their CD spectra can be significantly improved by taking into account the twist of β-sheets. Our new method, named Beta Structure Selection (BeStSel) generally performs better than any of the previously published algorithms.

6. The increased structural information provided by BeStSel makes the protein fold prediction possible. BeStSel provides extra information on the conformation of β-structures, which makes the protein fold prediction possible down to the topology level. This method is based on the analysis of structures in the Protein Data Bank.
Figure 3. By taking into account the orientation and twisting of β-sheets, the BeStSel algorithm provides sufficient information on the secondary structure to predict the fold of a protein from its CD spectrum.

7. **Circular dichroism spectroscopy is a suitable method to characterize the secondary structure of protein aggregates.**
With carefully executed CD or SRCD experiments, our novel method provides reasonable structural information on these protein forms that are, otherwise, difficult to study using other methods.

**Summary**

The structure-function paradigm of proteins has been revisited and undergone a significant transformation in the last years. The newly recognized diverse functions of various conformational states (unfolded state, various oligomeric and aggregated states including amyloid fibrils) of proteins enlightened a more complex view. The polypeptide chain of a given amino acid sequence may take up various conformational states stabilized by different intra- or intermolecular interactions playing roles in different physiological or pathophysiological processes. For the better understanding of the underlying molecular mechanisms, it is inevitable to gain structural information on the corresponding protein conformations and characterize the kinetics and thermodynamics of their formation.

In the first part of my PhD work I studied the thermal stability and dissociation process of the amyloid fibrils of β2-microglobulin (β2m), a protein responsible for the dialysis-related amyloidosis. I found that the reversible heat-induced depolymerization of β2m amyloid fibrils occurs by monomer dissociation from the fibril-ends and by the fracture of the fibrils. By measuring the thermal dissociation profile, we could study the effect of the environmental conditions on the stability of the β2m aggregates in a reproducible manner. By extending our studies on the amyloid fibrils of the K3 peptide fragment of β2m and on synuclein, a protein associated with Parkinson’s disease, we found that the method can be generally used for the investigation of the stability of protein aggregates.

The 20-residue Trp-cage miniprotein is an excellent model system to study the conformational transitions and aggregation of proteins. I studied the effect of phosphorylation on the structure of the Trp-cage. I found that the D9S(P) variant forms aggregates at low temperature different from amyloid fibrils resembling the collagen polyproline triple helix structure. Consistently with the experimental findings, an *in silico* structural model was built.
In the refinement of the model the formation of a stabilizing 16Arg-9Ser(P) salt-bridge was found which could be verified experimentally.

The previously available algorithms to estimate the secondary structure of proteins from the CD spectra were found incapable of predicting the structure of β-sheet rich proteins and amyloid fibrils. The spectral and structural diversity of the β-structures was thought to be an inner limitation of the technique. With a careful investigation of the protein CD spectra database, we showed that the CD spectral features of the β-structured proteins correlate with the orientation (antiparallel, parallel) and twisting of the β-sheets. We developed a novel method for the secondary structure determination of proteins taking into account the twist of the β-sheets. The method overperforms all the previous algorithms and, with the increased information content provided, it is capable of protein fold prediction as well.

Our Beta Structure Selection (BeStSel) method was successfully applied for the study of the secondary structure of various disease related protein aggregates. Moreover, the method provided unmatched performance on globular and membrane proteins and proteins with rare structural composition. We constructed a web server (http://bestsel.elte.hu) to make the method freely available for the research community as a general tool for the study of the secondary structure of any protein molecule by a simple and fast evaluation of single or multiple CD spectra.
My PhD thesis is based on the following publications


Further publications


International conferences


Kardos J, Wien F, Micsonai A. Accurate antiparallel and parallel β-sheet decomposition and fold prediction from srcd spectroscopy. Institute for Protein Research Seminar on Impacts of Supersaturation on Protein Science, Osaka, Japan, 18-19 June 2012


Micsonai A, Wien F, Kele Zs, Réfrégiers M, Kardos J. 3D2CD: Calculation of the CD spectra of proteins from their X-ray structures, 15th International Conference on Chiroptical Spectroscopy, Sapporo, Japan, 30 August – 3 September, 2015