A novel method for the secondary structure determination and fold recognition by CD spectroscopy and its application to investigate the structure-function relationship of aberrant protein aggregates and amyloid fibrils

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Summary

Circular dichroism (CD) spectroscopy is a widely used technique for the study of protein secondary structure. It is an inexpensive technique suitable for a fast structure verification of recombinant proteins, study mutants, conformational changes, protein folding and stability and protein-protein interactions. It is essential when NMR or X-ray crystallography are not applicable, because of the large size or the lack of crystals of the protein. The instrumentation of CD is well developped, however, despite all the efforts made in the last decades, the reliable CD spectrum analysis has been limited mainly to helical proteins. The difficulties arise from the structural and spectral diversity of the β-structures which have been considered as the inner limitation of the method. We have developed a new method by taking into account the twist of the beta-sheets which provides a reliable secondary structure determination for a wide range of proteins, able to distinguish parallel and antiparallel beta-sheet structure and predict the protein fold. Our treatment of CD spectra opens new perspectives in CD spectroscopy. In the present project we further developed our method and utilize its potential to gain more accurate and increased structural information from the CD spectrum and developed our method to a widely usable tool. It is especially useful to study the structure of membrane proteins, protein aggregates and amyloid fibrils and natively unfolded proteins. The method is freely accessible for the scientific community at the BeStSel webserver (https://bestsel.elte.hu) and used ~7000 times monthly from 61 countries. Besides method development, by using our method, we ana lyzed in details the secondary structure and fold of proteins of various structural classes from folded, globular proteins to natively disordered chains. Moreover, we characterized specific protein aggregates involved in the pathomechanism of degenerative diseases, such as Alzheimer's disease and studied the relationship between the structural properties and the physiological effects, such as citotoxicity. With these studies we addressed important scientific questions related to the stability and structure of proteins.

Results

1st year. One main goal of the project was to further improve our BeStSel algorithm for the protein secondary structure estimation from the CD spectra. The algorithm uses a reference dataset based on the SP175 SRCD dataset of the Protein Circular Dichroism DataBank. One direction for improvement is to extend the reference set with high quality CD spectra of proteins having known x-ray structures. In the first year, to this end, we expressed and purified several proteins rich in β-sheet structure, including β2-microglobulin, its mutants, and dinein light chain. We received various protein samples as gift from collaborating groups, such as immunoglobulin domains (β-sandwich), plasminogen activator (left handed antiparallel β-sheet), SH3 domains (right-hand twisted β-sheet) and human bileal acid-binding

protein (iBABP, β-barrel). We recorded the CD spectra of these proteins first on a conventional spectropolarimeter and then by SRCD at the SOLEIL Synchrotron Facility in France. The extended reference set was to re-optimize the BeStSel algorithm to provide (even) better basis spectra for spectral fitting. In collaboration with Orsolya Tőke, we analized the structure and structural transitions of the above mentioned iBABP, a perfect β-structured model protein for CD, by BeStSel and carried out MD simulations to model the structural transitions. The manuscript on the results has been accepted for publication in FEBS Journal (Horváth et al, FEBS JOURNAL 284 : 3637-3661. (2017) IF: 3.902)

Regarding the fold prediction, we have evaluated the newest CATH database and refreshed our single domain fold database. This way the number of folds in the database is increased from 1490 to 2737 at the level of homology. In parallel, we developed further the fold recognition module by using more sophisticated algorithms applying modern mathematics. The prediction power and reliability has been greatly improved.

We worked on the direct protein concentration detection from the CD measurement. In case of an available wide wavelength range spectrum (starting from 175 or 180 nm) a scaling factor resulting the least NRMSD upon the spectral fitting was proved to be a reliable indicator for the correct protein concentration. In the case of a narrower wavelength range (from 190 nm), the absorbances of the protein at 205 and 214 nm, which can be determined from the photomultiplier voltage, are good measures of the concentration. A module has been built for the web server to calculate the proper extinction coefficients from the amino acid sequences provided by the users.

2nd year. We improved further our fold prediction algorithm which predicts the protein fold from the secondary structure results of the analysis of the CD spectra. For this, the protein fold database was updated to use the data of the CATH 4.2 protein classification database. A new type of search method was introduced using the „weighted K-nearist neighborhood" algorithm. The prediction algorithm was included to the BeStSel webserver. After a presubmission iquery, the journal Nucleic Acids Research selected our proposal to submit a manuscript on the BeStSel webserver, which, after revisions, were accepted and published in June, 2018 (Micsonai et al., Nucleic Acids Res. 46, W315-W322 (2018), impact factor: 11.561). In agreement with the research plan of this K_16 grant, we added case studies to this paper including the structural analysis of the aggregation of Alzheimer's amyloid-β peptide, forming oligomers and amyloid fibrils. The number of the users of the BeStSel server in increasing steeply. Already more than 90,000 calculations were carried out from 57 countries and our original PNAS paper has been cited more than 260 times (see MTMT).

We continued the collection of protein CD and SRCD spectra to extend the reference database of BeStSel. Among other globular proteins, it is important to note C1q, the first component of the classical pathway of the complement system, which was isolated from serum. This protein includes a collagen-like tail with PPII structure. BeStSel does not account for PPII but this spectrum is important to investigate the limitations of the algorithm. However, the head domains of this protein exhibit mainly β-structure which is perfect to use as a reference for BeStSel. The plasmid of the single chain form of the human C1q head domain was received from French collaborators and we ourselves prepared its mouse variant. The human variant has an X-ray structure solved. The reference structure for the mouse protein was built by in silico modelling and MD simulations. The proteins were expressed in eukaryotic and in *E.coli* expression system as part of our other, National Brain Research Program project where we would like to use these proteins to interfere the function of the complement classical pathway.

Here, these head domains are important from the structural point-of-view having mainly relaxed and twisted β-structure. Their high quality CD spectra were recorded and processed. The main work on the functional study of the role of C1q in complement mediated synapse elimination, with a partial contribution from this K_16 project was published in *Proc. Natl. Acad. Sci. USA* (Györffy et al., PNAS, 115, 6303-6308 (2018), impact factor 9.504).

We studied the structure of amyloid fibrils and aggregates of various proteins by CD spectroscopy including α-synuclein variants, Supp-35 peptides, Aβ variants and artificial peptides and studied the problematics of their measurement in various aspects. We started the preparation of a "Protocol" manuscript on the methodology of the study of protein aggregation by CD spectroscopy. Most of the proteins used for these studies were expressed in our laboratory. With limited proteolysis, we studied the amyloid core of amyloid fibrils of α-synuclein. In collaboration with Prof. Yuji Goto (Osaka University, Japan) we studied the temperature and salt effects on competitive formation of amyloids versus amorphous aggregates which was published in the *Journal of Biological Chemistry* (JOURNAL OF BIOLOGICAL CHEMISTRY 293: 14775-14785. (2018) impact factor: 4.010).

We are working on the optimization of the 3D2CD algorithm which calculates the CD spectrum from the structure based on the "reverse use" of the BeStSel basis spectra. Related to this work, we calculated the CD spectra of model structures produced by extensive MD simulations using replica exchange method in collaboration with Dr. Lucio Colombi Ciacchi and co-workers (University of Bremen, Germany). The work, studying the conformational changes of a protein upon adsorption on silica surface has been accepted in *ACS Biomaterials Science & Engineering* (Hildebrand et al., ACS BIOMATERIALS-SCIENCE & ENGINEERING 4: 4036-4050. (2018), impact factor: 4.432).

We participated the Biophysical Meeting of the American Biophysical Society at San Francisco with two posters.

3rd year. We have set up the toxicity measurements of amyloid-β (Aβ) peptide (1-40) and (1-42) aggregates. Taking into consideration the pathophysiology of Alzheimer's disease, where the central nervous system is affected, we have focused on neuronal cells. Primary neuronal cell cultures were prepared from two areas of mouse embryo brain, the cortex and hippocampus. Cell viability assays were carried out and showed that amyloid-β peptides (both lengths) exhibit cytotoxicity. The toxicity is well expressed in the range of 2-20 μM Aβ concentration. We tested different immortalized cell lines as well, however, those cells were proved to be more stable and less sensitive for the toxic effects of Aβ. We found the mHippoE14 immortalized neuronal cell line of hippocampal origin suitable for toxicity assays. Aβ aggregates were prepared under various conditions (buffer, ionic strength, temperature, incubation time). The structural properties of the aggregates were studied by CD spectroscopy, thioflavin-T fluorescence, and electron microscopy. Toxicity measurements showed that the most toxic form of Aβ peptides was proved to be Aβ(1-42) small oligomer that was prepared at neutral pH, low temperature and low salt concentration by overnight incubation. Oligomer specific Aβ antibody showed a high signal for this sample. Monomers and fibrils exhibited less toxicity. The secondary structure analysis of the CD spectra of Aβ aggregates using our BeStSel method showed that oligomeric Aβ samples show significantly higher antiparallel-β and decreased parallel-β structure content compared to fibrillary Aβ. Considering the low seeding ability of the toxic oligomers compared to fibril seeds, these results revealed the characteristically different secondary structure of Aβ oligomers which are not compatible with the parallel-β amyloid core structure of fibrils. Based on the detailed structural data gained experimentally, our future goal is to build up an *in silico* structural

model of Aβ oligomers that can provide useful information to target oligomers as point of intervention in the pathophysiology of Alzheimer's disease.

We visited the SOLEIL Synchrotron facility twice in the 3rd year of the project and collected SRCD spectra of protein samples including amyloid fibrils and disordered proteins, as well, to extend our reference database to further improve the performance of the BeStSel method.

On the BeStSel method, we submitted an article to Methods in Molecular Biology which has been accepted for publication after revision but the publication of the book itself occurred in 2021 (András Micsonai, Éva Bulyáki and József Kardos. BeStSel: From secondary structure analysis to protein fold prediction by circular dichroism spectroscopy, METHODS IN MOLECULAR BIOLOGY 2199 pp. 175-189. , 15 p. (2021)).

We continued the structural and thermodynamic studies on amyloid formation in collaboration with Prof. Yuji Goto, Osaka University studying the effect of the environmental conditions and additives on the amyloid formation. On the polyphosphate induced amyloid formation of β2-microglobulin, we published Zhang et al., (Proc. Natl. Acad. Sci. USA, 116, 12833-12838 (2019) IF: 9.58). We also studied the amyloid formation of β2m at high temperature under agitation (Noji et al., J Biol Chem. 294:15826-15835 (2019) IF: 4.238).

We studied the effect of aromatic side chains on the far-UV CD spectra of β2-microglobulin and its Trp and Tyr mutants and on chymotrypsin containing 8 Trp residues. Using the mutant-wild type comparison, we determined the aromatic contributions to the CD spectrum. Using the 3D2CD tool (which is under development by us) we calculated the "aromatic free" spectra of the proteins and by subtraction from the experimental data, we also calculated the aromatic contributions. We are working out a method to have the aromatic contribution into account with the aim to improve the BeStSel algorithm for proteins rich in aromatic side-chains. We continued to study the problematics of CD spectrum calculation from the 3D structure and in collaboration with Prof. Ciacchi's group at University of Bremen we published Michaelis et al. (J. Phys. Chem. B 123:6694-6704 (2019) IF: 2.923).

Other goal of our project for this year was to start validate the usability of the X-ray structures of proteins as a structural reference for the solution CD spectrum. We started MD simulations on our reference proteins in explicit water to check if the average dynamic structure is different from the X-ray structures. The MD simulations of human I-BABP showed that the dynamics of the molecule is dependent on the protonation levels of the His side-chains. With these results we contributed to the article of Horvath et al. (Scientific Reports 9:4825 (2019) IF: 3.998).

Related to the present project in terms of CD spectroscopy measurements and MD simulations, we collaborated with the group of Dr. Gábor Pál on the investigation of the canonical loop-inhibitor scaffold relationships of serine protease inhibitors (Boros et al., J. Mol. Biol. 431:557-575 (2019) IF: 4.894).

We attended several conferences in the field of amyloid research and circular dichroism spectroscopy. Among these, József Kardos was an invited speaker at the 17th International Conference on Chiroptical Spectroscopy, Pisa, Italy, June 23-27, 2019.

4th year. We continued the structural-functional characterization of the aggregation of disease related peptides and proteins. We applied our methodology for the most important disease related systems, including the Alzheimers's amyloid-β peptide, α-synuclein, β2-microglobulin and polyQ.

The effect of Abeta(1-42) peptides produced by our lab and aggregated to form toxic oligomers were used in an *in vivo* mouse neurotoxocity model and pointed out the importance of the trpa1 receptor (Payrits et al., MECHANISMS OF AGEING AND DEVELOPMENT 189: 111268 (2020) IF: 4.304). In collaboration with Prof Yuji Goto (Osaka University), we have studied the aggregation mechanism of alpha-synuclein at its isoelectric point highlighting the role of solubility and supersaturation-limited mechanism (Furukawa et al., CURRENT RESEARCH IN STRUCTURAL BIOLOGY 2: 35-44. (2020). Amyloid formation of β2-microglubulin was studied under complicated realistic conditions in which β2-microglobulin coexists with its proteolytic fragments (Muta et al., BIOCHEMISTRY 2019, 58, 4925−4934. (2020) IF: 2.952).

We have further studied and compared the aggregation mechanism of two disease-related variants of β2-microglobulin, the wild-type one associated with dialysis-related amyloidosis and its single point mutant causing hereditary systemic amyloidosis. Preparing a variety of mutants we discovered that a synergistic effect of native monomer destabilization, amyloid fibril stabilization and an altered molecular interaction network is behind the dramatic differences between the two variants that might also explain the observed differences between in vivo disease mechanisms. We have presented our results at the conference “Towards a cure for amyloid diseases: a successful example of precision and translational medicine” in December 2019, Pavia, Italy.

Related to the present project in terms of CD spectroscopy measurements and the use of BeStSel on a wider conformational space, we collaborated with the group of Dr. Ágnes Tantos and Péter Tompa investigating the interplay of structural disorder and short binding elements in the cellular chaperone function of plant dehydrin ERD14. We have proven the protection effect of ERD14 on its substrate proteins under stress conditions (e.g. high temperature) by CD spectroscopy (Murvai et al., CELLS 9: 1856 (2020) IF: 6.6).

Using our methodology and experience in protein CD spectroscopy, we studied human and Plasmodium falciparum calmodulins’ structure and stability. By studying the aromatic region, we were able to distinguish the melting of the individual domains of calmodulin (Juhász et al., FASEB Bioadvances, 2020, DOI: 10.1096/fba.2020-00013).

We have continued the MD simulations of the reference structures used for the development of our BeSetSel method for a potential improvement of the secondary structure estimation.

József Kardos was invited to present the achievements of the research group in the analysis of protein structure by CD spectroscopy at the New York University of Abu Dhabi (United Arab Emirates) in February, 2020.

Unfortunately, in 2020, we could not visit SOLEIL Synchrotron for SRCD measurements because of the covid-19 pandemic. However, we have sent samples to France and the beamline scientist carried out some limited number of measurements for us.

Our Nucleic Acids Research paper on the new developments of BeStSel and case studies (Micsonai et al., Nucleic Acids Res. 46, W315-W322 (2018), received around 200 citations in two years and become highly cited in the Web of Science (top 1%) and for several months it won the “Hot Paper” title (top 0.1 %).

5th year. We continued the development of the BeStSel method and webserver. Unfortunately, we could not carry out any SRCD experiments at the SOLEIL French Synchrotron Facility in this period

because of the covid-19 situation. Our university did not allow foreign travels until the end of this summer. Our measurements at SOLEIL were re-scheduled to February, 2022. The half year further extension of this OTKA grant makes possible to realize this plan. Instead of SRCD spectra collection at SOLEIL, we carried out a thorough literature search and collected CD spectra of numerous proteins from published scientific papers. These spectra needed careful quality inspection and selection based on the available data on the conditions and experimental parameters. Our increased reference database further extends the conformational (folding) space covered and can be used as learning or test data. We took special attention to disordered proteins and proteins with highly twisted antiparallel beta-sheets to improve their distinction. We are working out a protocol to identify intrinsically disordered proteins (IDPs) from their CD spectra which method would complement and verify the results of the bioinformatics tools. Various types of spectral analysis and learning tools were tested and it turned out that the disorder can be efficiently predicted from the CD signal at three wavelength values, depending on the wavelength range. Spectra in the wide wavelength range (SRCD range) down to 175 nm, significantly increased the reliability of the prediction. A manuscript is under preparation and will be submitted to special issue on IDPs in Frontiers in Molecular Sciences.

The BeStSel method reached 1000 citations on the two published papers (Micsonai et al., PNAS, 2015 and Micsonai et al., Nucleic Acids Research, 2018) revealing the great interest from the scientific community. The new developments will be online at the webserver by the end of this year.

Our work on the characterization of the two, amyloid disease-related variants and further mutants of beta2-microglobulin were completed. The wild-type and hereditary D76N mutant of the protein cause two distinct type of amyloidosis. By using CD spectroscopy and a variety of biophysical techniques, we found that, relative to WT β2m, the exceptional amyloidogenicity of the pathogenic D76N β2m variant is realized by the deleterious synergy of diverse effects of destabilized native structure, higher sensitivity to negatively charged amphiphilic molecules (e.g., lipids) and polyphosphate, more effective fibril nucleation, higher conformational stability of fibrils, and ele-vated affinity for extracellular components, including extracellular matrix proteins. This work is part of the PhD work of Éva Bulyáki who will defend her thesis early in 2022.

Using our methodology and expertise in CD spectroscopy, we contributed to several collaborative works in the IDP and amyloid/protein aggregation field. We studied the cellular chaperone function of intrinsically disordered dehydrin ERD14 protein where the structure forming tendencies of different regions of the protein were identified by CD spectroscopy examining several fragments and truncated variants of ERD14. The advantage of the use of CD spectroscopy was that we could study large number of variants under various conditions which could not be realized by NMR spectroscopy. This work was published in the International Journal of Molecular Sciences (Murvai et al., Int. J. Mol. Sci, 2021, 22: 6190 (2021), IF: 5.923). Collaborating with Prof. Yuji Goto (Osaka University, Japan) in studying the structural and thermodynamic background of amyloid formation, our results were published in Communications Biology (Noji et al., Comm. Biol., 4, 120 (2021), IF 6.268). In a joint work with the group of András Lukács (University of Pécs) we studied the C-terminal tail extension of myosin 16 and found that it acts as a molten globule, including intrinsically disordered regions, and interacts with the N-terminal ankyrin (Telek et al., J. Biol. Chem. 297, 100716 (2021), IF: 5.157).

József Kardos was invited to give a lecture at the conference “Amyloid School- Budapest” in November, 2021.

Last 6 months. In the last six months, we could finalize several works that was delayed because of the covid-19 pandemics. We have prepared several manuscripts and successfully had them accepted in Q1 and D1 journals. We published our work on the amyloid formation of the two disease-related variants of β2-microglobulin, the wild-type one associated with dialysis-related amyloidosis and its single point mutant causing hereditary systemic amyloidosis, in the journal Biology (Bulyaki et al., BIOLOGY-BASEL 10: 1197 (2021), IF: 5.168). Preparing a variety of mutants, by using CD spectroscopy and a variety of biophysical techniques, we found that, relative to WT β2m, the exceptional amyloidogenicity of the pathogenic D76N β2m variant is realized by the deleterious synergy of diverse effects of destabilized native structure, higher sensitivity to negatively charged amphiphilic molecules (e.g., lipids) and polyphosphate, more effective fibril nucleation, higher conformational stability of fibrils, and elevated affinity for extracellular components, including extracellular matrix proteins. We discovered that a synergistic effect of native monomer destabilization, amyloid fibril stabilization and an altered molecular interaction network is behind the dramatic differences between the two variants that might also explain the observed differences between in vivo disease mechanisms. Éva Bulyáki has submitted her PhD thesis entitled "The role of the pathogenic Asn76Asp mutation in the amyloid formation of β2-microglobulin", which was mainly based on this work. She defended her thesis in the spring of 2022.

The BeStSel method reached 1400 citations on the two main papers (Micsonai et al., PNAS, 2015 and Micsonai et al., Nucleic Acids Research, 2018) revealing the great interest from the scientific community. We continued the development of the BeStSel method and webserver. Because of the covid-19 situation, our measurements at SOLEIL Synchrotron Facility were re-scheduled to the last, extended half year of the OTKA project, to February, 2022. We continued the collection of the SRCD spectra of various states of amyloidogenic proteins and natively disordered polypeptide chains, which helped the further development of the BeStSel webserver. In November, 2021, we sumitted a proposal to the journal Nucleic Acids Research to contribute to its 2022 webserver issue, which was accepted. We submitted the manuscript in March, 2022 and the reviewers found it acceptable after minor revision (Micsonai et al., NUCLEIC ACIDS RESEARCH 50: 90-98. (2022), IF: 19.160).

We carried out a thorough literature search and collected CD spectra of numerous proteins from published scientific papers. These spectra needed careful quality inspection and selection based on the available data on the conditions and experimental parameters. Our increased reference database further extended the conformational (folding) space covered and can be used as learning or test data. We took special attention to disordered proteins and proteins with highly twisted antiparallel beta-sheets to improve their distinction. We have worked out a protocol to identify intrinsically disordered proteins (IDPs) from their CD spectra which method complements and verifies the results of the bioinformatics tools. Various types of spectral analysis and learning tools were tested and it turned out that the disorder can be efficiently predicted from the CD signal at three wavelength values, depending on the wavelength range. Spectra in the wide wavelength range (SRCD range) down to 175 nm, significantly increased the reliability of the prediction. The method was introduced at the BeStSel webserver (https://bestsel.elte.hu) as a new function. The corresponding manuscript was submitted to the special issue on IDPs in Frontiers in Molecular Biosciences and accepted for publication in March, 2022 (Micsonai et al., FRONTIERS IN MOLECULAR BIOSCIENCES 9: 863141 (2022), IF: 6.113)

Overview, expoitation, and conclusions

The project was successfully achieved. We published 21 papers with a cumulative impact factor of 120.61 and presented our results in 21 posters for the community. Several young researcher have

participated in the project gaining professional experience. András Micsonai and Éva Bulyáki PhD students have prepared and defended their PhD thesis based on their work in the project. During the project years, the BeStSel webserver became popular and widely known for analysis of the CD spectra. The two main papers on BeStSel has received more than 1200 independent citations and became highly cited (top 1%) in the Web of Science. ~7000 analysis are carried out on the server monthly, from 61 countries of the world. Becoming the leader CD spectrum analysis method, BeStSel has raised the interest of a Japanese instrument company for a stand-alone version to build the software in their instrument and use it off-line. We have already signed a contract with them.

The project was highly supported by international collaborators from France, Japan and Korea. We have to note that our Hungarian-French bilateral TÉT grant and a TÉT grant to Japan helped the collaboration with the French partner in the SOLEIL and the Japanese in Osaka. Our K_16 project greatly benefited by this TÉT grants.

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