Journal of Alzheimer's Disease 6 (2004) 99–105
IOS Press

Discussion

Live discussion: Protein folding and neurodegeneration: Biophysics to the rescue?[1]

Live discussion held 30 April 2003 on the Alzheimer Research Forum, featuring David Teplow.
http://www.alzforum.org/res/for/journal/teplow/teplow_transcript.asp
Participants: Nikolay Dokholyan (*University of North Carolina, Chapel Hill*), *Charlie Glabe* (*University of California, Irvine*), *Dave Holtzman* (*Washington University, St. Louis*), *Alexei Koudinov* (*Russian Academy of Medical Sciences*), *Aleksey Lomakin* (*MIT*), *Nathan Oyler* (*NIH*), *Nathalie Paganini* (*Neurochem, Inc.*), *Dave Teplow* (*Harvard Medical School*), *David Thirumalai* (*University of Maryland*), *Rob Tycko* (*National Institute of Diabetes and Digestive and Kidney Diseases, NIH*).

Gabrielle Strobel: Exactly what is meant by "natively unfolded" or "intrinsically disordered"? Does it mean that α-synuclein (and also Aβ) is sort of flopping about unstructured in its physiological state and then prodded into folding by a binding partner? This would fundamentally change our conventional thinking about protein structure-function relationship, no?

Dave Teplow: Gabrielle, I will take a stab at your question and let the real biophysicists correct me. When we are taught biochemistry, we see proteins in their physiologically active forms. These forms are their native forms. The reason why Peter Lansbury (MIT) and others used the term "natively unfolded" for α-synuclein and other proteins, especially peptides, is that they do not exist, to a significant degree, in the same kind of stable structure that larger native proteins like myoglobin do. This does not change our view of protein folding in the least.

Nikolay Dokholyan: I do not think there is a contradiction to the conventional thinking of protein structure-function relation. Some functions are performed by disordered protein structural parts.

[1] Note: The transcript has been edited for clarity and accuracy.

Rob Tycko: Most of the liquid-state NMR data, obtained on monomeric Aβ, indicate that the peptide by itself is dynamically flexible in solution. In the fibril state, the peptide becomes highly ordered (at least from residue 10 on). The degree of conformational order in the prefibrillar aggregates is not yet known. I think the issue of structural order in the prefibrillar states is an especially interesting one at this time.

Alexei Koudinov: Rob, the same may be true when the protein has its natural environment. We showed previously that Aβ is bound to lipoproteins such as apolipoprotein [1]. We and others also showed that the LP lipid environment affects its secondary structure [2].

Rob Tycko: Alexei, I am sure you are right.

Alexei Koudinov: There is a good link to oligomers from the apolipoprotein story. Many apolipoproteins have a tendency to oligomerize and polymerize out of the lipid environment, to self- and cross-associate [3]. Furthermore, some apolipoproteins form different types of amyloidoses, like SAA, ApoA1, etc. Structurally apolipoproteins depend on their amphipathicity, and Aβ is also an amphipathic molecule as a recent Science paper [4] mentions and the article by

ISSN 1387-2877/04/$17.00

Charlie Glabe [5] showed. There are several studies that show association with lipoproteins arrests toxicity of Aβ. Unfortunately, last year's oligomer article by Walsh et al. [6] and the latest Science article [4] miss this consideration.

Dave Teplow: Rob, your work in the fibril area has been superb. I agree with you that we also want to examine the earlier, less stable structures. The question is how to reduce the ensemble of structures to allow this examination.

Gabrielle Strobel: Rob, how would it be best to study the structural order of those states?

Rob Tycko: We have not done these measurements yet, but it should be possible to assess structural order in prefibrillar aggregates by doing solid-state NMR measurements, for example, on freeze-trapped solutions.

Dave Teplow: Yes, but what kind of data can be produced with an equilibrium mixture?

Rob Tycko: We do not know yet. If there is a very broad distribution of behavior, the data might be hard to analyze. Dave Templow, you are the expert on preparing oligomeric Aβ. What would you suggest in the way of sample preparation protocols to maximize the abundance of a single aggregated form.

Dave Teplow: We are actively working on this question. I have no "recipe" at this point, but I have two avenues of exploration: 1. Alterations in the primary structure to stabilize particular assemblies, and 2. alterations in solution conditions to achieve the same goal. Also, Hilal Lashuel (Harvard Medical School) has published work on a chemical compound that stabilizes protofibrils. Maybe these attempts could work to establish a standard protocol.

Gabrielle Strobel: Nikolay, do we know which functions of Aβ are performed by a disordered state versus an ordered state?

Nikolay Dokholyan: Not that I know of.

Charlie Glabe: The prefibrillar aggregates/oligomers/micelles/protofibrils/ADDLs are different from fibrils. For example, in Aβ, the C-terminus is buried in oligomers and exposed in fibrils, based on fluorescence quenching.

Rob Tycko: Charlie, when you say the C-terminus is exposed in fibrils, how close to the C-terminus do you mean?

Charlie Glabe: Residue 40 in Aβ40; strange, huh? Residue 38 is also less spin-coupled and more mobile.

Gabrielle Strobel: Are there no crystal or solution structures of Aβ because it is dynamically flexible? How else can we get structures of these things? Could they be bound to binding partners and then crystallized?

Nathan Oyler: Alternatively, has anyone tried dynamic light scattering on solutions of Aβ as a function of time to follow the aggregation?

Charlie Glabe: A pH of 9 and lower salt concentration help stabilize oligomers, but they are not completely stable. They keep on aggregating.

Dave Teplow: Nathan, we have published extensively in this area. Aleksey Lomakin can answer all of your questions about this.

Aleksey Lomakin: I am listening.

Rob Tycko: This is why we would want to freeze-trap the oligomer samples, to prevent progressive aggregation.

Dave Teplow: Rob, what level of homogeneity would you require in the freeze-trapped SSNMR experiment?

Rob Tycko: One might be able to crystallize Aβ somehow, but the conformation in the crystalline form would probably not be relevant to the conformation in oligomeric or fibrillar forms.

Charlie Glabe: Still, though, oligomers are stable enough for spectroscopy. You have hours to days to work with.

Dave Teplow: Charlie, same question. How pure do the samples have to be to determine from what species the signals come?

Rob Tycko: Again, we do not really know until we try. If, for example, a given segment of Aβ is helical in oligomers ranging from 10 to 50 molecules in size, we could still tell it was helical even if we had this kind of distribution.

Gabrielle Strobel: An intriguing paper earlier this month [7] suggested that the structural requirements for binding in a protein complex can overwhelm any intrinsic folding preferences within a natively unfolded protein and essentially drive its folding. Does this sound crazy?

Rob Tycko: No, it is not crazy.

Dave Holtzman: Along Gabrielle's question, are there techniques, say, by labeling Aβ radioactively or fluorescently, combined with biophysical techniques, to study Aβ structure in a more complex physiological solution that mimics the brain's extracellular space and its varied proteins and lipids?

Charlie Glabe: Dave, obviously, the more homogeneous the samples, the better, but you can get some idea of the structural homogeneity from the spectra.

Alexei Koudinov: Dave, this would be very important, as the solutions used seem to be too artificial to represent the *in vivo* reality.

Rob Tycko: Alexei, I think one should not necessarily dismiss data obtained under "artificial" conditions without having a specific reason to do so. A lot of scientific progress has come about from *in vitro* experiments.

Alexei Koudinov: You are right. . . . We just have to interpret them with caution and think of the next step in approaching the *in vivo* situation.

Gabrielle Strobel: If binding partners were involved in determining the relevant conformation, perhaps age-related changes in those other (physiological) binding proteins could have as much to do with altered Aβ folding, and then aggregation, as Aβ accumulation, per se.

Dave Teplow: You have suggested a very viable etiologic process.

David Thirumalai: There apparently are a number of examples where protein is natively unfolded and gets ordered only upon function, i.e., interaction with other proteins. This suggests that it is crucial to understand what unfolded means and what unfolded means in a finite concentration of proteins.

Aleksey Lomakin: I always thought unfolded means no longtime correlation in structure.

Nikolay Dokholyan: Resolving a structure of a single Aβ protein may provide only limited insight on "native" Aβ conformation. Since it may not be stable in any of the conformations, one would get only one possible structure out of many.

Alexei Koudinov: Nikolay, it seems important to define what concentration range we are talking about. In case we make a 4–20 ng/ml Aβ in PBS (or some other way buffered), will we have Aβ polymerized into fibrils? Any comments? A note: a 4–20 ng/ml Aβ concentration was reported previously to be in CSF.

Dave Thirumalai: I think there is considerable debate on what unfolded means in infinite dilution, let alone in the presence of other proteins/peptides.

Nikolay Dokholyan: I would define natively disordered as those peptides that do not have free energy minima of more than a couple of kBT (energy of thermal fluctuation). Of course, that does not imply that some parts of the peptide are structured. . . . Alexei, of course concentration affects the environment.

Dave Holtzman: Alexei, that is correct that the concentration of all Aβ species in the CSF in humans ranges from $\sim$10–30 ng/ml.

Alexei Koudinov: Dave, there are several papers on Aβ concentration. Despite some variability, the trend is in the range you specify. Thanks.

Dave Teplow: Question for all: What do we do with Charlie's latest findings? Charlie, are you trying to define the epitope and, therefore, the "active" structure recognized by your antibody?

Charlie Glabe: Dave, yes, it would be nice to know the structure of the oligomers and what the epitope is, but it is not so easy. We (Ral Langen and I) have made some progress in defining the structure of the large spherical oligomers or micelles. We are trying to map the antibody-binding site by EPR spectroscopy.

Rob Tycko: Charlie, could you summarize your latest findings? I have a copy of your paper, but have not had a chance to read it yet.

Charlie Glabe: Rob, basically all soluble oligomers have a common structural feature that an antibody recognizes regardless of sequence. This is basically like Ron Wetzel's (Drexel University) results with fibrils.

Gabrielle Strobel: All, you can read up on Charlie's paper, and comments by Dominic Walsh (Harvard Medical School), Harry LeVine (University of Kentucky), and others at the Alzheimer Research Forum [8].

Nikolay Dokholyan: I think another important outcome of Charlie's work is that immunoreactivity of Aβ40 oligomer was not different from Aβ42 oligomers, but delayed.

Gabrielle Strobel: Can you explain the implication of this, Nikolay?

Nikolay Dokholyan: It may be the consequence that sequence was not important for forming the oligomers, but was important for their stabilization, affecting equilibrium association.

Gabrielle Strobel: Charlie, please correct where I went wrong: I seem to recall that your synthetic Aβ antigen was about 95 percent pure and you immunized with it 10 times? I find it astounding and fascinating that that produced such an interesting specificity in the polyclonal serum-a misreading of your paper?

Charlie Glabe: The peptide is about 95 percent homogeneous. The rabbit has been boosted over 20 times now and we do not see "normal" Aβ antibodies being produced-same thing for mice and dogs.

Rob Tycko: I thought peptide/antibody interactions usually depend on specific side chain interactions, not just backbone conformation. Charlie, what are your possible explanations for this result that an antibody can recognize a class of structures independent of sequence?

Charlie Glabe: It must recognize the conformation of the peptide backbone. That is the only thing that they might have in common.

Alexei Koudinov: For me, the Science article antibodies imply that similarity of amyloidogenesis pathways for amyloid proteins. I take it as a major take-home message (and it is in the title, as far as I remember). This importance can be of secondary value in AD, though, in case other mechanisms are of primary value. For us, such primary disease change is a cholesterol homeostasis failure.

Dave Teplow: Charlie, have you attempted to determine the KD dissociation constant for the antibody? This would help answer the question posed by Rob. A specific interaction would likely produce more avid binding.

Charlie Glabe: Dave, we have not measured it accurately, but it is sufficiently high that you can do "normal" things with it like affinity purify it, IP, histochemistry.

Nikolay Dokholyan: Charlie, do you know the structure of the antibody?

Charlie Glabe: We would like to know. We tried to crystallize the Fab from rabbits, but no luck. We are trying to get a monoclonal.

Nikolay Dokholyan: What about in complex with the oligo?

Rob Tycko: If you cannot solve the Fab/peptide structure by crystallography, you can get information about the bound peptide conformation from NMR. We have done this for HIV-related peptide/antibody complexes.

Charlie Glabe: Nikolay, yes, ideally, but maybe with a small peptide, too.

Nikolay Dokholyan: NMR would do, too.

Dave Thirumalai: Question for experimentalists: I do not see clean experiments that probe oligomerization kinetics as a function of protein concentration. Are there studies of this type around?

Gabrielle Strobel: The hydrophobic microenvironment of Aβ, i.e., the membrane composition immediately around it, appears to influence its secondary structure. In AD, age-related local alterations in the content of cholesterol, its esters, and other lipids might alter AβPP trafficking through the cells' various membrane compartments, and AβPP processing. Is there a way to model this environment in structural and aggregation studies?

Alexei Koudinov: Gabrielle, your note on the importance of membrane microenvironment should be tightly linked to Aβ extracellular appearance as lipoprotein-associated molecule and raise a question of greatest value: What are the ways of intracellular traffic of Aβ?

Gabrielle Strobel: Nathalie, can you tell us about compounds Neurochem has to interrupt Aβ aggregation?

Nathalie Paganini: At the moment, Neurochem is conducting a phase II clinical trial on NC-758 to investigate the safety and tolerability of this drug candidate in patients with mild to moderate Alzheimer's disease (AD). NC-758 was specifically designed to modify the course of the disease by interfering with the association between glycosaminoglycans and Aβ.

Gabrielle Strobel: Is this Cerebril?

Nathalie Paganini: Gabrielle, Cerebril is also targeting Aβ in patients who have had lobar cerebral hemorrhage. I have to add that Cerebril is also at the stage of clinical phase II trials.

Rob Tycko: At what point in the Aβ aggregation process does this glcyosaminoglycan/Aβ association occur, or what is the most important point in this process, do you think?

Gabrielle Strobel: Nathalie, what is the mechanistic difference between the two compounds?

Nathalie Paganini: We are talking about the same compound.

Gabrielle Strobel: I see.

Nathalie Paganini: In the phase II trial for Cerebril, safety and tolerability are the two primary endpoints at the moment.

Dave Teplow: Following the discussion about Cerebril, it may be interesting for people to express their opinions abut which assemblies should be targeted, i.e., which assemblies are the key pathogenetic effectors in AD.

Charlie Glabe: Target any and all assemblies. If you inhibit any transition, it will be useful for structural studies.

Rob Tycko: I am not a physician or even a real biologist, but is it not established that the inflammatory response to the presence of fibrils plays at least some role in the progression of AD?

Dave Teplow: Charlie, you just lost your Mulligan rights. How about sticking your neck out a little more.

Gabrielle Strobel: Yes, Rob, this is how I understand it, too. And Charlie, would it not be dangerous to block some transitions when we do not know where the neurotoxicity starts? We might be accumulating toxic intermediates. . . .

Charlie Glabe: My point is to find all inhibitors because they will all have some use. If you are talking about clinically, then you can sort out the clinically useful ones later.

Gabrielle Strobel: Dave Thirumalai, for those who have not yet read your excellent review, can you summarize what plausible scenarios you think govern amyloid aggregation?

Dave Thirumalai: I think we made several points. The one most relevant to scenarios is the following: The pathways depend on the nature of the monomer. If the monomer is "unfolded" natively (Aβ, β-synuclein, etc.), then we argue that interaction-driven ordering is the first event prior to forming a "critical nucleus." Otherwise, one has partial denaturation followed by interaction-driven aggregation. In both instances, there are multiple pathways. But this is taken for granted nowadays.

Charlie Glabe: Even *in vitro* with synthetic Aβ, it seems that you can elongate fibrils by addition at ends, and you can form them by "annealing" protofibrils or maybe even adding oligomers on the ends.

Aleksey Lomakin: When you inhibit some structures, Aβ should go somewhere else. If we are not addressing production or sinks, we can only talk about redirection of pathways.

Nikolay Dokholyan: So the question is: How do we measure toxicity to characterize the pathways?

Alexei Koudinov: Gabrielle, it will also be important to find the differences between similar motifs in oligomers and some membrane/lipid-bound proteins

(including apolipoproteins in LP environment), so we would not target normal pathways.

Rob Tycko: I know it is dangerous to talk about unpublished data, but we know from recent experiments on Aβ that there are at least two distinct fibril nucleation processes that lead to fibrils with distinctly different morphologies and somewhat different solid-state NMR spectra (and hence, somewhat different molecular conformations within the different morphologies). So the amino acid sequence does not uniquely determine the molecular conformation within amyloid fibrils.

Dave Teplow: Nikolay's question gets back to that of enriching particular forms of Aβ.

Nikolay Dokholyan: Rob, did you observe two different pathways under the same conditions? From simulations of SH3 domain proteins, we observed that they undergo cooperative transition under one condition, while there are unpopulated intermediates under other conditions.

Dave Teplow: Rob's comment is reminiscent of the strain question in prion disease. Here, different prions can induce different folds in the same endogenous protein.

Dave Thirumalai: I think that differences in pathways might manifest in "strains," as well. This is relevant to diseases, I believe.

Gabrielle Strobel: Dave, what do you mean by strains in the context of AD?

Dave Thirumalai: Not really in AD, but in prions.

Rob Tycko: Subtle differences in fibrillization conditions lead to different fibril morphologies, reproducibly. It seems that the key difference may be the presence or absence of interfaces (e.g., air/water interface). We plan to test for differences in fibril toxicity in cell cultures.

Dave Thirumalai: Even in AD, there can be variations in the final product that depend on pathways, as Rob just mentioned.

Gabrielle Strobel: We are talking biophysics. It would be natural if many in the neurodegeneration field were facing technical and methodological barriers to engage seriously in studies of the folding of Aβ, β-synuclein, tau, and other suspects. What could Alzforum do to help bring those barriers down and bring more people into this line of research? Ideas?

Dave Teplow: Rob, Dan Kirschner's (Boston College) work in this area has revealed that primary structure differences also result in substantial differences in morphology, for example, producing short fibrils or sheets instead of long 10-nm fibrils.

Alexei Koudinov: I would like to call on all to check out last Friday's Science paper on nanotubes and nanowires made of Aβ dipeptide [9]. This interesting effect should be studied in greater detail.

Gabrielle Strobel: Susan Lindquist (Whitehead Institute, MIT) has a similar paper about prion nanotubes, and she commented briefly on the Gazit et al. paper on Alzforum [10].

Rob Tycko: In our case, the primary structure, peptide concentration, pH, temperature, ionic strength are all the same, but we still get different morphologies from different types of nucleation.

Dave Holtzman: This strain effect in prion protein diseases may be analogous to protein interactions with Aβ and the risk for AD. Apolipoprotein E isoform effects may be an example, via slight alterations in Aβ interactions. Perhaps there are genetic alterations at other genes/proteins that modify AD risk via altering Aβ conformation/pathways of folding.

Dave Thirumalai: I think an interesting question is how do different morphologies emerge and how are they related to heterogeneity of interactions (environment/sequence, etc.). This can be answered computationally as well as using toy models. Apolipoprotein E, then, may be the protein X of the prion field.

Gabrielle Strobel: We have reached the end of the hour. Thank you, Dave and everyone, for coming, discussing, and speculating so openly.

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