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Structure and sequence determinants of aggregation investigated with molecular dynamics

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1. ABSTRACT

Spontaneous self-assembly and amyloid formation are a general property of many polypeptides and the information controlling these processes is encoded in the sequence. This determines the form and structural features of the interacting partners that regulate the properties of the final aggregates. Understanding the correlations between sequence, structure and dynamics in peptides and proteins at an atomistic level of resolution still represents one of the grand challenges of modern biological chemistry. In this context, computer simulations represent a valuable approach to understand recognition and spontaneous self-organization, processes that cannot be directly observed experimentally. Herein, we will discuss cases illustrating the extent to which simulations can be used to understand the self-organization properties of natural and designed amyloidogenic peptide sequences. The simulations provide evidence for the influence of specific interactions with well defined stereochemical constraints on fibril formation. The results from our and other groups suggest that simulations can be applied to detect the critical physico-chemical determinants of a certain process and can be helpful in the design of new chemical systems and experiments.

2. INTRODUCTION

The investigation of peptide-protein aggregation and amyloid formation has attracted great interest from scientists across a wide spectrum of disciplines, ranging from medicine, biology and pharmacology to chemistry, biophysics and physics. Initially, this was due to the observation that the deposition of the insoluble and intractable aggregates, defined as amyloid fibrils, was a hallmark of a broad range of degenerative diseases in humans. Many of these pathological conditions are caused by the failure of a specific peptide or protein to correctly fold to its native functional conformation. Once this happens, the otherwise soluble proteins or protein fragments start to aggregate and precipitate from solution forming insoluble plaques, and the originating conditions are generally referred to as *protein misfolding* (*or protein conformational*) *diseases*. More than twenty unrelated protein-misfolding diseases are currently known and include for instance type II diabetes, Parkinson's, Alzheimer's, Creutzfeld-Jacobs disease, and several unrelated forms of amyloidosis (1-6). The proteins involved share no apparent similarity in terms of sequence, structure and function. Yet, the aggregation process is always associated with a structural transition from the "folde" state

of the proteins into a common, collapsed *β*-sheet-rich structure (4). This process is often thermodynamically irreversible and the large supramolecular aggregates eventually formed are very stable (7). More recently, mounting evidence has suggested that the toxicity of the fatal neurodegenerative diseases may be caused by the intermediate oligomers that form on pathway to the final aggregate, in addition to the mature fibrils (8). Understanding the physicochemical determinants of these processes is thus an issue of primary importance in the biomedical context, in particular in view of the development of new drugs for the pharmaceutical treatment of the above mentioned pathologies.

Moreover, the ability to form amyloid aggregates has been observed also in a large number of proteins that are not related to any diseases. Aggregation in these cases has been induced under particular solvent, pH and temperature conditions (9-11). Interestingly, the non-pathogenic supramolecular complexes display the same structural features as their disease causing counterparts, being characterized by the presence of high percentages of *β*-sheet motifs. The possibility to control the formation of supramolecular peptide and protein based structures through the proper choice of solution conditions has opened up the possibility to use peptide-based aggregates for nanotechnological applications (12). Ellis-Behnke *et al.* have used a designed self-assembling peptide to create a nanofiber scaffold generating a permissive environment for axons not only to regenerate through the site of an acute injury but also to knit the brain tissues together (13). This approach was tested with success in animal models. Smith and coworkers showed that insulin fibrils share mechanical properties even with inorganic materials such as a strength comparable to that of steel (14). Moreover, Reches and Gazit showed that it is possible to direct peptide aggregation to the formation of nanotubes which were used to cast metallic nanowires (15).

Fibrils and peptide aggregates possess thus attractive properties for future technological applications.
One of the most remarkable aspects of the fibril formation process is that the common structural properties are shared among sequences of high diversity. Amyloid fibrils share a number of similarities that include a common cross-β diffraction pattern typical of an elongated stack of β-strands perpendicular to the fibril axis and β-sheets parallel to the axis. These facts have led to the view that the propensity to form amyloid fibrils may be a general property of the polypeptide backbone (16), suggesting that at infinite time any protein solution above a critical concentration will eventually undergo structural transition into the aggregated state (7). In contrast to this view, several studies have shown that the propensity of a certain polypeptide chain to aggregate depends dramatically on amino acid composition (17-20) and that, therefore, side-chains actually influence the formation and stability of amyloid aggregates (17-21). However, despite some progress (22), the role of the sequence is not clear yet, mainly because of the lack of detailed structural information. Indeed, the high degree of complexity of aggregation, combined with the fact that initial oligomers are metastable and short-lived, makes atomic-level experimental structural data difficult to obtain.

In contrast, obtaining this level of resolution is already possible for computational and theoretical approaches. Simulations can provide insight into the properties of the complicated free energy landscapes of aggregating peptides shedding light on the monomer dynamics and oligomer formation mechanisms, and can be used to analyze complex interactions linked to the presence of amyloidogenic profiles in different sequences and structures (23-27). The use of short and simplified peptide systems has been particularly important in this field (25): eliminating the complexity due to the presence of whole proteins has allowed researches to pinpoint the effects of the perturbation of single properties on aggregation. Moreover, the small size of certain systems has proved useful to catch the role of fine chemical details related to variation of structures and properties in experimental observations.

Most of the studies based on all-atom models have dealt with the simulation of single peptides or with the study of preformed oligomers with geometries compatible with available experimental information. A few recent examples have tried to extend the use of MD methods to the *ab initio* investigation of the mechanism and kinetics of multiple chain aggregation from random initial configurations (26).

In this paper we will review the use of theoretical methods based principally on all-atom molecular dynamics (MD) to investigate the conformational properties of short peptides involved in amyloidogenic processes, to investigate their possible modes of interactions in preformed fibril models and the role of sequences in their stabilization, and the possible use of these methods to develop anti-amyloid leads. Moreover, we will introduce a new approach developed in our laboratory to extend the reach of *ab initio* simulation of oligomer formation beyond what is currently accessible by normal all-atom MD.

At this point, it is worth underlining that most of the theoretical work has concentrated on short sequences, while in many cases fibril formation involves whole proteins. In the latter case, there is little consensus on whether native-like domains are conserved or completely refolded in the final amyloid state and whether these domains may retain their catalytic activity. We will also try to address this problem by discussing recent evidence showing that, at least in designed amyloid-like fibrils of Ribonuclease A (RNase A), enzymatic activity could be conserved in the aggregate suggesting partial conservation of native-like structures in the aggregate.

3. CONFORMATIONAL PROPERTIES OF AMYLOIDOGENIC PEPTIDES

3.1. Conformational properties of amyloidogenic peptides

The conformational free-energy landscapes (28-30) of different sequences involved in aggregation

processes are characterized by a rugged profile, reflecting the multiplicity of states available to the peptides at room temperature.

In particular, some of these states may be particularly prone to form intermolecular contacts promoting the association with other monomers leading to the final fibrillar state (31).

It is difficult to characterize the conformational properties of these states using conventional NMR and X-ray crystallography techniques, because of their heterogeneity, rapid interconversion and tendency to precipitate from solution. All-atom computational simulations can in contrast provide a direct view of the main representatives of the conformational ensembles, in different environmental conditions, such as temperature, pH, charge state or capping groups on the terminals *etc.*

Understanding the properties of aggregating peptides in solution, in terms of the chemical details responsible for the relative weight of conformational states and underlying their assembly properties, could be fundamental in designing new drugs. One could in fact design molecules (peptidomimetics or synthetic molecules based on defined scaffolds) that mimic those conformational properties and feature chemical functionalities able to interfere with the intermolecular recognition process at the basis of fibril formation.

In this context, the full length Aβ peptide, responsible for the deposition of plaques associated to Alzheimer's disease, was studied with extensive sampling in solution using long timescale, all-atom MD simulations. Flock and coworkers (32) simulated the peptide in aqueous solution (explicit water) at different temperatures, pH values and with different charge states of the N- and C-termini to study the mechanisms associated with conformational transitions preceding fibrils formation. The starting structure of the peptide corresponds to the a-helical conformation determined in organic solvent by NMR spectroscopy. The simulations in explicit water are characterized by a rapid loss of the native structure in favor of β-rich structures which display a high structural stability. Moreover, β-sheet rich regions expose a hydrophobic patch located on the edge of the forming β-hairpin to contact with the aqueous solvent, suggesting a possible anchor point for the molecular recognition of other monomers in analogous conformations.

The full length peptide was also studied was studied with all-atom MD simulation by other authors (33). The monomer was studied using aqueous and apolar solvents at 300K in five simulation.

The analyses of the data showed results in line with those reported above, with a sensitive reduction of the alpha-helical content at the C-terminal in favor of a random coil conformation. In particular, four small β-sheets were formed by four glycines at positions 25, 29, 33 and 37, one glycine for each sheet.

To test the role of the four glycines in conformational selection, mutations to alanine residues, which are helix promoters in water, have been studied on them in four different simulations. The results indicated an increase and stabilization of the alpha helix structure and the absence of beta-sheets. These evidences suggested that the sequence stretch 25-37 was important for the formation of a β-sheet in aqueous solution.

In addition, one more simulation was run at a higher temperature (400K) to speed up conformational changes. In this case, the conformational dynamics of the peptide was found to be very similar to the ones seen before: the transformation from alpha helix to random coil presented an intermediate phase in which the peptide adopted a helix/beta-sheet mixed conformation.

The Aβ (1-40) peptide was also studied in a bilayer formed by dipalmitoyl phosphatidylcholine molecules to understand the peptide behavior in membrane environment as residues 29-40 belong originally to the transmembrane domain of the Amyloid-beta Precursor Protein. In this situation, it was shown that the peptide structure did not have β-sheet elements while there was a strong helical presence, furthermore during the simulation the peptide moved from the hydrophobic region to the hydrophilic water molecules (33).

A recent Replica Exchange Molecular Dynamics study by Garcia and coworkers (34) has also concentrated on the role of peptides Aβ (1-40) and Aβ (1-42) in the early steps of aggregation. The authors characterized the conformations of Aβ (1-40) and Aβ (1-42) in water by using a combination of molecular dynamics and measured scalar coupling constant data from NMR experiments. Through REMD simulations they first found that classical forcefields could reproduce the NMR data quantitatively when the sampling was extended to the microseconds time-scale. Using the quantitative agreement of the NMR data as a validation of the model, they extended their study to compare the conformational ensembles of the Aβ (1-40) and Aβ (1-42) peptide monomers. The analysis confirmed the existence of structured regions within the otherwise flexible Aβ peptides. In particular, the C terminus of Aβ (1-42) resulted more structured than that of Aβ (1-40). The formation of β-hairpin in the sequence IIGLWGGVVIA involving short strands at residues 31-34 and 38-41 reduced the C-terminal flexibility of the Aβ (1-42) peptide and was hypothesized to be responsible for the higher propensity of this peptide to form amyloids.

Shorter fragments of the full length peptide were investigated experimentally to identify the minimal sequence determinants of aggregation. Sequences spanning region 25-35, 10-35, 21-30, 12-28 and 16-22 were shown to form fibrils which had supramolecular properties comparable to those formed by the full length sequence. The reduced dimensions of the segments make them attractive models to investigate the relationships between conformational behavior and aggregation properties.

In a notable case (35), the conformational space accessible to the Ab (21-30) peptide was investigated by using replica exchange molecular dynamics simulations in explicit solvent. Conformations belonging to the global free energy minimum from simulation resulted to be in good agreement with reported NMR structures. These conformations were characterized by a bend motif spanning the central residues V24-K28. This bend was stabilized by a network of hydrogen bonds involving the side chain of residue D23 and the amide hydrogens of adjacent residues G25, S26, N27, and K28, as well as by a salt bridge formed between side chains of K28 and E22. The higher energy states of this peptide were compact and retain a similar bend topology. The persistence of structure in the higher energy states was supposed to account for the resistance of the peptide to protease degradation and aggregation, even at elevated temperatures.

Wei and Shea explored the conformational free energy landscape of the Aβ (25-35) using replica exchange molecular dynamics in both pure water and in HFIP/water cosolvent (36).

The use of the replica exchange methodology allowed to perform an efficient sampling of the conformational space of the system. Moreover, this technique was well suited to study the conformational space of peptides with energy landscapes dominated by many basins separated by high barriers. Results show that the peptide preferentially populates a helical structure in apolar organic solvent, while in pure water the peptide adopts collapsed coil conformations and to a lesser extent β-hairpin conformations. These analyses indicated that the Aβ sequence is extremely flexible and populates a wide range of secondary structures that can be "fine-tuned" by the solvent.

Comparison of the results obtained using different solvent shows that that the backbone groups of the β-turn region able to form hydrogen-bond are more exposed to water than the remaining residues of the peptide, suggesting that the β-turn in Aβ (25-35) is a candidate site for initiating aggregation, forming the hydrogen bond motifs parallel to the long axis of the fibril. In the organic cosolvent mixture, where the peptide does not aggregate, all residues show low backbone H-bonds exposure to solvent.

From these results it is evident that one important aspect of the early steps of fibrillogenesis is the environment-dependency of the conformational behavior of peptides, according to the hypothesis that conformational transition of proteins from a soluble and functional form into a β-rich structure presumably occurs in the partially denaturing environment of a specific cellular compartment (37).

Similar conclusions have been reached by Knecht and coworkers (38), who studied the conformational behavior of fibrillogenic fusion peptide B18 in different environments using Molecular Dynamics simulations.

Replica exchange simulations were also used by Baumketner and Shea (39) to sample the conformational states of Aβ (10-35) peptide. The fragment possesses many of the amyloidogenic properties of its full-length counterpart. Under physiological temperature and pressure, their simulations revealed that the Aβ (10-35) peptide did not possess a single unique folded state. Rather, this peptide displayed a conformational ensemble made up of a mixture of collapsed globular states in rapid dynamic equilibrium with each other. This conformational ensemble was shown to be dominated by random coil and bend structures with insignificant presence of a helical or beta-sheet structure. The 3D structure of Aβ (10-35) was defined by a salt bridge formed between the side-chains of K28 and D23. This salt bridge was also observed to be important for the stability of full length Aβ fibrils and these simulations suggested that monomeric conformations of Aβ (10-35) could contain pre-folded structural motifs promoting rapid aggregation of this peptide.

All atom molecular dynamics were also used to investigate the properties of the Aβ (12-28) fragment (24, 40). This fragment was studied in both monomerical and oligomerical configurations (dimer and tetramer) at different temperatures (295K and 320K). For the monomers the starting conformations chosen were helical or extended (Figure 1). The oligomers were built by selecting the most populated representative conformation obtained from the statistical analysis of the structures obtained from the monomer simulations.

The results showed that, in the case of the monomers, the secondary structure shifted in a few nanoseconds from an a-helix or extended conformation to bent conformations. This transition was stabilized by a salt bridge between Lys16 and Glu22 or Asp23 and also by the packing of hydrophobic side chains of residues 17-21. This led to the formation of a plane of five contiguous hydrophobic residues that could act as a possible hook to bind other monomers in analog conformations, acting thus as a fibrillization seed.

This hypothesis was supported by the analysis of the internal energy profile which presented an increment due to the breaking of the helix and then a decrease during the formation of the bent structure.

The bent conformation was then used as a starting point for other molecular dynamics simulations. The results obtained define a clear transition of the secondary structure to a stable beta hairpin.

In the beta hairpin monomer hydrophobic side chains were exposed to the polar solvent but the conformation was stabilized by salt bridges between Val12-Lys28 and Lys17-Asp23 and by several backbone-backbone hydrogen bonds (Figure 1b). The beta hairpin structure was consistent with several observations based on X-ray diffraction analysis of similar peptides (4, 41, 42).

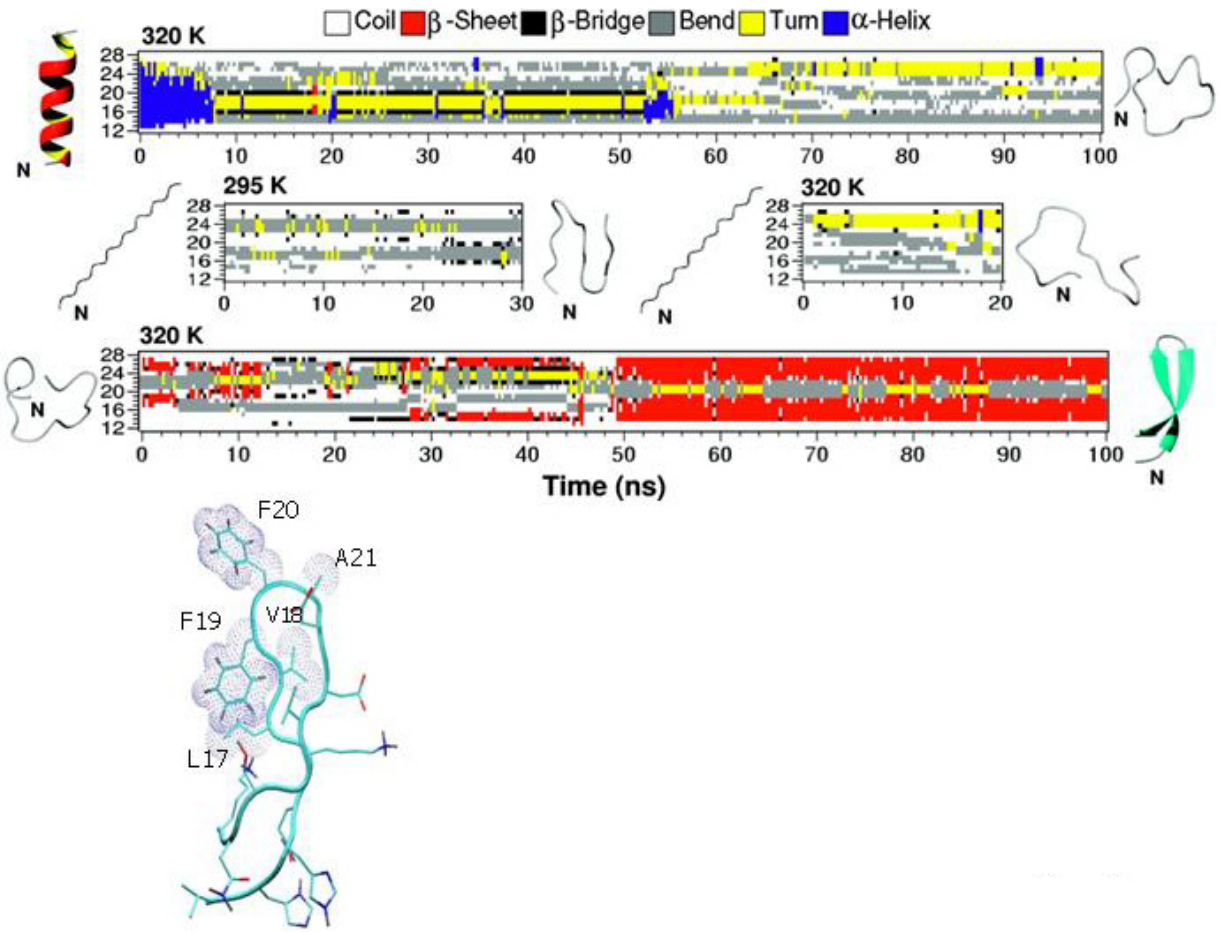

Figure 1. Time dependent evolution of the structure of Abeta (12-28) in water, from the helical NMR structure determined in membrane mimicking solvent. Secondary structure elements are assigned according to the dssp criterion. The isolated structure displays the disposition of the hydrophobic side chains in contact with the solvent.

The beta hairpin structure was thus chosen as the original building block to model of dimers and tetramers in order to test which residues and which kind of interactions were important in the formation of oligomers. Analysis executed on these simulations underlined the presence of hydrophobic, hydrogen bonds and Coulombic interactions involving residues from number 14 to 28 that have a major role in the stabilization of the structure assumed by oligomers, as it was shown in small peptide models (18) and in other theoretical studies (24, 43).

A protocol based on shape complementarity was used to generate an assortment of possible dimer structures for the Ab (10-35) peptide by Tarus and coworkers (44). The structures generated based on shape complementarity were evaluated using rapidly computed estimates of the desolvation and electrostatic interaction energies to identify a putative stable dimer structure. The potential of mean force associated with the dimerization of the peptides in aqueous solution was computed for both the hydrophobic and the electrostatic driven forces using umbrella sampling and classical molecular dynamics simulation. The comparison of the two free energy profiles suggested that the structure of the peptide dimer is determined by the favorable desolvation of the hydrophobic residues at the interface. Molecular dynamics trajectories originating from two putative dimer structures indicated that the peptide dimer is stabilized primarily through hydrophobic interactions, while the conformations of the peptide monomers could undergo substantial structural reorganization in the dimerization process. The authors found the phi-dimer to constitute the ensemble of stable Ab (10-35) dimer. In particular, the expulsion of water molecules at the interface was identified as a key event, similarly to the oligomerization process of Abeta (16-22) fragments, underlining the importance of peptide-peptide and peptide-water interactions.

Fragments derived from Aβ peptide derived peptides were also studied using Monte Carlo simulations.

For example the Aβ (16-22) peptide has been analyzed using systems with one, three or six chains in order to represent fibrils oligomers (45).

Evaluating the secondary structure content, it was found that in the case of the monomer the peptide

assumed mainly a random coil conformation with different temperatures, while in the case of oligomers, with low temperatures values, there was a preference to self-assemble into aggregates showing a high beta-strand content. In particular for the hexamer, the beta structure was present at higher temperatures compared to the three stranded oligomer, as reflected also by the specific heat curve. Furthermore, both parallel and antiparallel conformations were found during the simulations, with a major preference for the antiparallel beta-sheet (this was obtained ignoring the charged side chains of Lysine and Glutamate at the termini of the peptide). The authors hypothesized the driving force for the aggregation to be due to the geometry of backbone-backbone hydrogen bonds, steric effects and the distribution of hydrophobicity along the chains. Klimov and Thirumalai, investigating the same peptide using MD simulations, hypothesized Coulomb interactions to be determinant for the aggregation mechanism (21).

All these studies suggest that the conformational transitions and the consequent aggregation process is driven by hydrophobic interactions due to side chains contacts but also to hydrogen bonds and electrostatic interactions. These results, on the other hand, also show that thorough analysis of the available conformational space for even short sequences is a challenging task. To overcome this problem, one could use better sampling method, or employ direct methods that provide information on the energy landscape from the analysis of the free-energy minimum structure.

In the former case, Higo and co-workers (46) used a powerful conformational sampling method, multicanonical molecular dynamics, to obtain an accurate free-energy landscape of the 25-residue segment of Aβ, Aβ (12-36), in explicit water over a wide temperature range.

From the analysis of the constructed free energy landscape at 300 K in water, this peptide was shown to populate five conformational clusters, each with its own characteristic tertiary structure: two β-structure clusters, one helical-structure cluster, and two random-structure clusters. Computed β-rich structures and the experimentally determined in-fibril structures showed however some differences. In particular, the experimental β-structures is stabilized by the inter-peptide strand-strand H-bonds in fibril, whereas the computed b-rich structures is stabilized by intra-peptide ones. These results suggest that in a multichain system there is a large free-energy change due to rearrangement of the intra-polypeptide H-bonds toward the inter-polypeptide H-bonds: all the intra-polypeptide H-bonds should be broken to convert Aβ to the in-fibril form.

A check of the capability of multicanonical molecular dynamics simulations to reproduce a reliably determined experimental structure was performed by Higo and co-workers (23). NMR experiments showed that the Aβ (12-36) peptide could adopt a unique tertiary structure in a 40% (v/v) 2,2,2-trifluoroethanol (TFE)/water solution. The peptide was simulated in the same solvent condition, starting from a random conformation. Simulations correctly folded the peptide to the experimental NMR structure. An interesting results of these simulations is that the shape of the free energy landscape of this peptide was found to be strongly dependent on temperature and an abrupt transition was observed at 325 K. Above 325 K, the overall shape was funnel-like, with a global minimum coinciding exactly with the NMR structure. Below 325 K, the landscape became more rugged, with the emergence of new conformational clusters connected by low free-energy pathways. These results suggest that this behavior can be common to most peptides, since potential energy barriers become more difficult to overcome at lower temperatures, leading to a rougher free-energy landscape.

A different approach to obtain insight into the free-energy profile of a certain sequence and its relationship to aggregation propensity is based on energy decomposition analysis which allows to identify which residues are the most relevant for the stability and folding of the protein in selected environments (47).

Using this approach Colombo and co-workers (48) studied the misfolding of the Prion protein (PrP^c) to its scrapie form PrP^{sc} which is related to a group of fatal neurodegenerative diseases in humans and animals. They analyzed the trajectories obtained by means of long timescale, explicit solvent, all-atom simulations of the protein, obtaining information on the determinants of its (de)stabilization properties.

Results indicated that the stabilization energy of PrP^c is concentrated in a core involving parts of helices H1 and H3. Misfolding starts with a spreading of the stabilization energy over helix H2, which destroys the native stabilizing core. The original energy distribution is finally restored in the final β-rich conformation. This clearly indicated that the prion sequence can adapt to two different folds of comparable free energy. This type of analysis can be used as a relatively rapid diagnostic tool to distinguish the conditions which promote misfolding from those which do not.

Extending this approach, Colombo and co-workers comparatively analyzed the trajectories for the Prion protein with those of the Doppel protein (49).

This comparison is particularly interesting since the Prion protein and the Doppel protein share a similar native fold despite a very low sequence homology, but the Doppel protein lacks the scrapie isoform.

Results show that Doppel possesses a core of amino acids whose interactions concentrate about 30% of the overall stabilization energy, whereas PrP^c only 20%. As a consequence, the stabilization core of Doppel is stable and it is conserved under misfolding conditions while the PrP^c's core is disrupted, so that this protein can access conformations prone to aggregation.

These results indicate that this approach can be used to overcome the limitations of all-atom molecular dynamics simulations, which currently cannot provide direct thermodynamic information about the folding and

misfolding of proteins of the size of the two studied in this work.

3.2. Investigating the role of sequence in oligomer stability. the simulation of preformed fibril models

The *ab initio* study of ordered-fibril formation is still out of reach for all-atom MD simulation methods. Exceptions exist to this rule. For instance, using a simplified implicit solvent treatment, Gsponer and coworkers studied the aggregation of the heptapeptide GNNQQNY, from the N-terminal prion-determining domain of the yeast protein Sup35, with 20 molecular dynamics runs for a total simulation time of 20 microseconds. The simulations generated in-register parallel packing of GNNQQNY beta-strands, consistent with x-ray diffraction and Fourier transform infrared data. Information on the properties of the energy landscape could be obtained from such an approach: the authors identified the presence of enthalpic barriers to the in-register conformation originating from out-of-register interactions. The parallel β-sheet arrangement was found to be favored over the antiparallel because of stacking interactions of the tyrosine rings and hydrogen bonds between amide groups. (50).

However, the study of pre-formed model oligomers still represents a viable method to investigate the effects of sequences on the stability of small aggregates possibly acting as aggregation seeds. Clarifying these factors on simplified model systems, that have been characterized experimentally, is fundamental to our understanding of the physicochemical determinants of amyloid stabilization.

Dimers are the simplest example of oligomeric nucleus, and it is known that dimer formation is critical in Aβ assembly.

Huet and Derremaux (51) studied the effects of mutations for Aβ dimers by high temperature all-atom molecular dynamics simulations of Aβ (9-40), Aβ (9-42) peptides and their Flemish variants (A21G) starting from their fibrillar conformations. The effects of the point mutation on Aβ dimers were found to be length-dependent and, moreover, the structures and dynamics of the Aβ (9-42)-A21G peptide could not be extrapolated from those of Aβ (9-40)-A21G and *vice versa*.

The point mutation actually affected the populations of the intramolecular and intermolecular salt bridges in both peptides, destabilized the β-sheets in Aβ (9-40) but not in Aβ (9-42) and increased to a higher extent the flexibility of the central hydrophobic cluster of both proteins.

The authors concluded that these effects were likely to slow down the formation of higher-order species and could explain the reduced aggregation rate of Aβ fibrils containing the Flemish disease-causing mutation, suggesting that the final organization of the fibrils depends in a specific way on the sequence.

Nussinov and coworkers (52) studied the effect of point mutations on oligomers of the DFNKF peptide derived from human calcitonin and its mutant, DFAKF, by performing parallel-tempering molecular dynamics simulations in explicit water with a total simulation time of 500 ns. Parallel-tempering MD allowed to explore the energy landscape of amyloidogenic peptide more effectively than constant-temperature simulations, running a series of simulations at various temperatures, ranging from low to high temperature. The simulations have pointed toward the important role of the Asn residue in amyloid formation. Actually, the assembly of a parallel β-sheet was promoted by the Asn-Asn interactions which acted like a glue, sticking the DFNK strands together. When the Asn was mutated to Ala, the tendency to form stable parallel-β-sheet trimers was noticeably reduced.

The use of minimal models that recapitulate the structural properties of supramolecular aggregates proved particularly useful in combination with the study of designed amyloidogenic sequences. Using *de novo* designed systems with minimal dimensions could provide a great wealth of insight into the determinants of peptide aggregation (18, 53). The control one can have on the sequence combined with the absence of interferences from large protein domains on the final structure, allowed researchers to pinpoint the fine physicochemical determinants of peptide aggregation. In this context, our group concentrated on one of the first and best characterized examples of amyloidogenic model systems, the short sequence STVIIE (25). We carried out a systematic all-atom molecular dynamics study of the effects of the charged state and single point mutations of this peptide on the stability of polymeric β-sheet structures similar to those assumed to be present in amyloid fibrils.

The simulations have provided evidence that the net charge of the molecule and the relative disposition and orientation of hydrophobic side-chains played an important role on β-strand arrangement and twist of amyloid β-sheet oligomers (Figure 2). The analysis of the effect of some mutations on the position dependence of β-sheet polymerization were in good agreement with experimental results. In particular, we observed that sequence positions that provide the higher number of stabilizing interactions for a given β-strand arrangement are more sensitive to mutation. Moreover the results pointed out that specific intermolecular recognition determined by aromatic stacking interactions is needed to obtain an ordered β-sheet complex that may lead to the growth of fibrils whereas a simple hydrophobic collapse could not determine the final ordered supramolecular structure (Figure 2). Ordered hydrophobic/aromatic interactions, were shown to provide the additional energetic stabilization counteracting the disaggregating effect of charges. This fact could be particularly important during the fibril growth phase, in which the number of side-chain hydrophobic interactions grow as the fibril grows, resulting in a net cooperative stabilization of the aggregate.

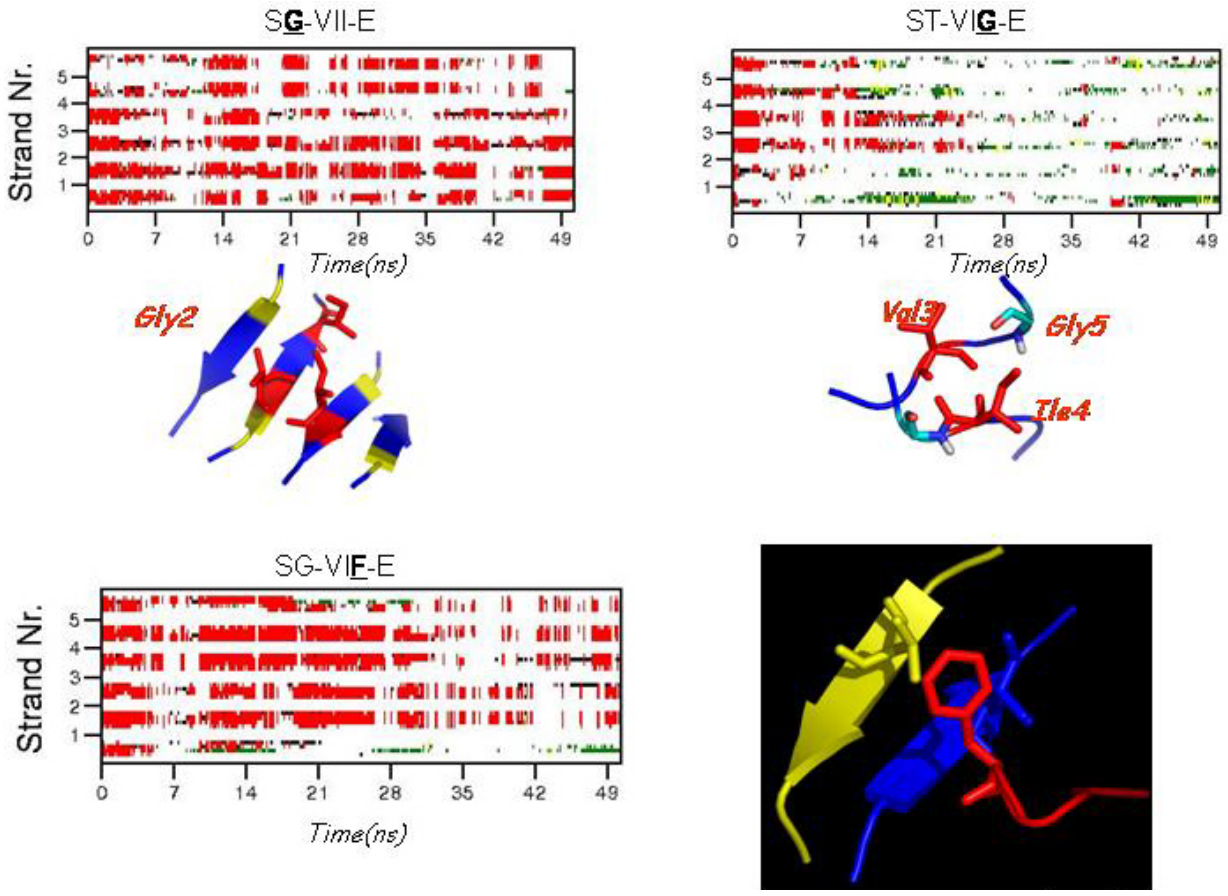

Figure 2. Time evolution of the ordered beta-sheet content for mutants of the aggregating STVIIE sequence. SGVIIE forms fibrils, STVIGE does not. The presence of Phe at position 5 strengthens fibril formation. The stabilization or destabilization as a function of the mutation is visible from both the secondary structure evolution graphs and from the representative structures+

Interestingly, the simulations provided details on the structural flexibility of the six-stranded nucleus, showing that it could give rise to different three-dimensional arrangements, preserving the antiparallel β-sheet arrangement. In the case of non-amyloidogenic sequences, the model nucleus could simply disaggregate or form amorphous complexes (25).

These results were confirmed (54) by a study of a short amyloidogenic peptide fragments from the medin polypeptide. Twelve independent 50 ns long molecular dynamics simulations in explicit water of this peptide and several single-point mutants were performed, considering a three-stranded polymeric β-sheet as the elementary unit from which amyloid fibrils can assemble. This study provided additional evidence for the importance of intermolecular aromatic interactions in the stabilization of short model peptides: the presence of the aromatic Phe residue at well-defined positions and with well-defined stereochemical orientation in space was shown to play key role for the stabilization of the three-stranded amyloidal unit.

The results of these simulations were in line with what observed also by Nussinov and co-workers, who studied the amyloid-forming peptide GNNQQNY from the yeast prion Sup-35 using explicit water molecular dynamics simulations (55). They performed simulations of various sizes and arrangements of oligomer seeds of the wild-type peptide and its mutants to study its stability and dynamics. The results showed that the stability of the oligomers increased dramatically with the increase in the number of strands, suggesting that the minimal nucleus seed for GNNQQNY fibril formation could be small. Moreover, mutant simulations showed that a further important role in determining the stability of oligomers is the correct geometrical matching of the side chains via intersheet interactions.

Analogous conclusions were reached by Rohrig and co-workers (56) who studied the structure and stability of oligomers of different sizes of the fragment 16-22 of the Alzheimer peptide using atomic-detail molecular dynamics simulations with explicit solvent. They observed that only the large β-sheet aggregates were stable, probably due to better hydrophobic contacts and better shielding of backbone-backbone hydrogen bonds from the solvent.

Han and Wu performed a long-time (1.7 μs) molecular dinamics simulation on the hexamers of wild-type Aβ(16-35) and its mutants (57).

Results indicated that the charge state could influence the amyloid formation process; actually the amyloid-like structures in the wild-type oligomers were destabilized by the solvation of ionic D23/K28 residues, which are buried in the fibrils. This suggested that the burial of these charged sidechains may determine a barrier to aggregation in the early stage. The role of electrostatic interaction in amyloid b-protein oligomer formation was studied by Yun and co-workers (58) through discrete molecular dynamics simulation. Electrostatic interactions promoted formation of larger oligomers in both Aβ(1-40) and Aβ (1-42). Moreover simulations suggested that C-terminal region played a key role in the formation of Aβ(1-42) oligomers and the importance of this region increased in the presence of electrostatic interactions. These results suggest that C-terminal region of Aβ (1-42) could be a target for inhibitors to prevent oligomer formation. A detailed analysis on the relative contributions of different structural elements to the stability of Aβ fibrils was performed by Buchete and Hummer (59, 60).

A similar approach in which hypothetic arrangements were built to study the aggregation properties of small peptides was applied to investigate the fibril organization of polyglutamine repeats (61).

Starting from a rigid nanotube-like conformation of model peptides rich in glutamine, the authores obtained a new conformational template consisting of a super-helical arrangement of flat β–sheet segments linked by planar turns or bends. The new system, remodeled using this template, showed high dynamical stability in simulations.

An interesting result has been obtained by Liepina and co-workers (62) who carried out molecular dynamics simulations of two-strand and ten-strand β-sheets constructed from two peptides corresponding to the diverging turn of two homologous Abl-SH3 domains, DLSFMKGE (MK, from drosophila) and DLSFKKGE (KK, from humans). Comparing the simulations, they observed that systems constructed from MK were more stable than systems constructed from KK, suggesting that MK systems is prone to aggregation and the KK systems were not. These results helped explain why most SH3 domains possess two conserved residues at the diverging turn, which probably have a protective role against aggregation.

Zhang and co-workers used molecular dynamics simulations to evaluate the effect of environment on the elongation of the PrP (106-126) fibril (63). Experimental data demonstrated that PrP (106-126) can form fibril under acidic solution conditions but the mechanism of fibril elongation and how acidic environment influences the formation of the fibril remain still unclear. Starting from an initial structure of PrP (106-106) generated by quantum chemical methods, they performed several simulations adjusting the pH values by either altering the protonation state of the residues or adding hydronium ions or hydroxyl ions. Using this method they observed that the peptide became a new element of the fibril when it changed to β-shee*t al*ong the edge strands, indicating the fibrils propagates by elongation at both ends.

From these results it is evident that studies concerning the characterization of short sequence peptides that retain all the molecular information needed to undergo fast and efficient fibril formation represent a very useful reductionist approach to shed light on the specific molecular details that underlie the aggregation process. This type of reasoning can clearly be extended to study the aggregation and self-organization properties of designed sequences. This could be particularly useful for nanostructure design.

One possibility is to use building bloks derived from repeat proteins. In this case a repeat is duplicated and assembled following the native geometry to create a stack. Nussinov and co-workers studied the left-handed β-helix repeat. This represented an excellent candidate to be used as building block to construct fibrillar or tubular nanostructures without the need to perform many structural manipulations. Using molecular dynamics simulations they constructed and tested 17 β-helix based systems providing a first screen that could limit the range of constructs to be tested experimentally (64).

Using a model based on the self-assembly of motifs taken from *Escherichia coli* galactoside acetyltransferase they produced a very stable nanotube in molecular dynamics simulations. In a subsequent study they modified this model by changing the charge distribution in the inner core of the system and testing the effect of this change on the structural arrangement of the construct. Results demonstrated that it was possible to generate the proper conditions for charge transfer inside nanotubes based on assemblies of specific motifs (65). The substitution of natural residues by engineered, constrained residues that restrict the conformational freedom and have favorable interactions, geometry and size was also shown as a valuable means to enhances the stability of β-helix nanotubes (66).

4. A NEW APPROACH FOR THE AB INITIO SIMULATION OF SPONTANEOUS PEPTIDE AGGREGATION

The use of all-atom methods for the study of the conformational properties of single chains in relation to their aggregation outcome, and the simulation of preformed minimal fibril models have provided a great deal of information on the roles of sequence, structure and conformational dynamics on the stability and formation of peptide amyloids.

The self-organization of multiple peptide chains into ordered aggregates is however an inherently very complex problem: the free-energy landscape that a system composed of different chains has to explore is enormously vast and so is the number of degrees of freedom that should be

considered to completely characterize that landscape. As a consequence, the typical timescales required for spontaneous aggregation from totally random placements of the sequences are out of reach for all-atom MD simulation methods. We tried to address this problem through the application of a mixed simulation scheme in which a preliminary coarse-grained Monte Carlo (MC) analysis of the free energy landscape is used to identify representative conformations of the aggregates and subsequent all-atom molecular dynamics (MD) simulations are used to analyze in detail possible pathways for the stabilization of oligomers.

The time advancement in this approach is obtained by a selection of possible aggregated oligomeric structures from an simulated annealing MC analysis of the evolution of systems consisting of several copies of a certain peptide, using a very simplified statistical interaction potential. The peptides are represented with a bead-on-chain model, considering each residue as a Cα based centroid. The peptides can move and rotate on a discrete cubic grid with 3 A spacing between consecutive points. The formation of aggregates from a completely random initial orientation is identified by monitoring the sudden drop in energy as a function of temperature corresponding to the phase transition in which partially ordered structures start to form from the otherwise disordered ensemble. This simplification allows to adequately sample the otherwise enormously vast conformational space available to the system.

The necessary simplifications also prevent the structures obtained from being as accurate as desired. Thus, the idea of feeding them hierarchically into a more accurate method that samples less effectively but with a higher degree of chemical and physical detail can be exploited. The structures identified at the transition point are thus reconstructed in full atomistic detail and refined with all-atom, explicit solvent MD simulations. In this context, MD simulations started from the sampled conformations are expected to have significant advantages over, for example, those starting from fully random peptide orientations, which can be affected by insurmountable (on the MD time scale) lag-phases. At this stage, in parallel to the protein folding case, it is not possible to provide the actual quantification of the time-advancement in the absence of clear, general, experimentally derived characterizations of the fibrillation mechanisms, and also in the absence of the definition of a general reaction coordinate. The benefits of the present strategy can be ascertained only through the comparison of the degree of order, β-sheet content, three dimensional organization and side chain interaction patterns of the explored trajectories comparing them to the experimentally determined structure of the fibril forming peptide chosen as a test system. In this case, the GNNQQNY fibril forming peptide was chosen as a model. This peptide represents the first example of fibrillogenic sequence for which an atomic-level high resolution structure was obtained (67, 68). The structure revealed unique features along with expected properties. The final fibril shows a hierarchical organization based on different types of stabilizing interactions. The basic unit of the assembly is a pair of β-sheets separated by a dry interface. Each β-sheet is formed by parallel strands, which are orthogonal to the fiber axis. In addition to the classical hydrogen bonds between backbone atoms, the β-sheets are stabilized by hydrogen bonds between polar side chains (the polar zipper) (67). At the dry interface, the sidechains of residues N2, Q4 and N6 are tightly interdigitated with the corresponding residues of the opposing sheet (steric zipper) (67). All of these properties represent an attractive target for benchmarking theoretical aggregation studies and molecular simulations in particular.

The results obtained from the combined approach are consistent, from the structural point of view, with experimentally derived structures and mechanistic considerations. Analysis of our mixed MC-MD trajectories suggests a hierarchical picture of the assembly process, in which parallel β-sheets form easily in many instances and are in general not disrupted even at high temperature conditions. Moreover, they are mostly characterized by an in-register organization of interchain hydrogen bonds. Preformed parallel β-sheets can act as templates in the aggregation process to accelerate fiber formation. Once the first seed is formed, more strands can pack on it mainly through van der Waals and H-bonding interactions involving the N and Q side chains, giving rise to the second level of organization. Optimal packing is achieved when the peptides forming the second plane are antiparallel to the first one. This mechanism leads to the formation of the dry interface, with exclusion of water molecules from the space between the two layers. It is worth noting that the pattern of interactions in the second stage is less specific than the one determined by the formation of in-register hydrogen bonds observed in the first level, as can be expected considering the flexibility of the amidic side chains of the interdigitating residues. The non-specificity of van der Waals interactions in the dry interface may lead to polymorphism in the structures of the starting aggregates, which can also be reflected in the amyloid fibril polymorphism observed for several sequences and strictly dependent on the solution conditions. Examples of the sampled structures in simulations containing different numbers of monomers are presented in Figures 3, 4.

Following the all-atom dynamical evolution of the initial stages of the aggregation process of the GNNQQNY peptide, becomes possible starting from several, different configurations obtained by a preliminary coarse exploration of the complex free energy landscape. The all-atom trajectories highlight the formation of ordered, mainly parallel, β-sheet aggregates. Remarkable stability is associated to the dimeric structures even at high temperatures. Moreover, the analysis of the chemical interactions, secondary structure evolution, and 3D conformational evolution determined by the simulations has resulted in dynamical information on the first stages of the aggregation process. Interestingly, the stable structures emerging from the simulations reproduce the main structural features observed for the peptides in the X-ray structures obtained by Eisenberg and coworkers.

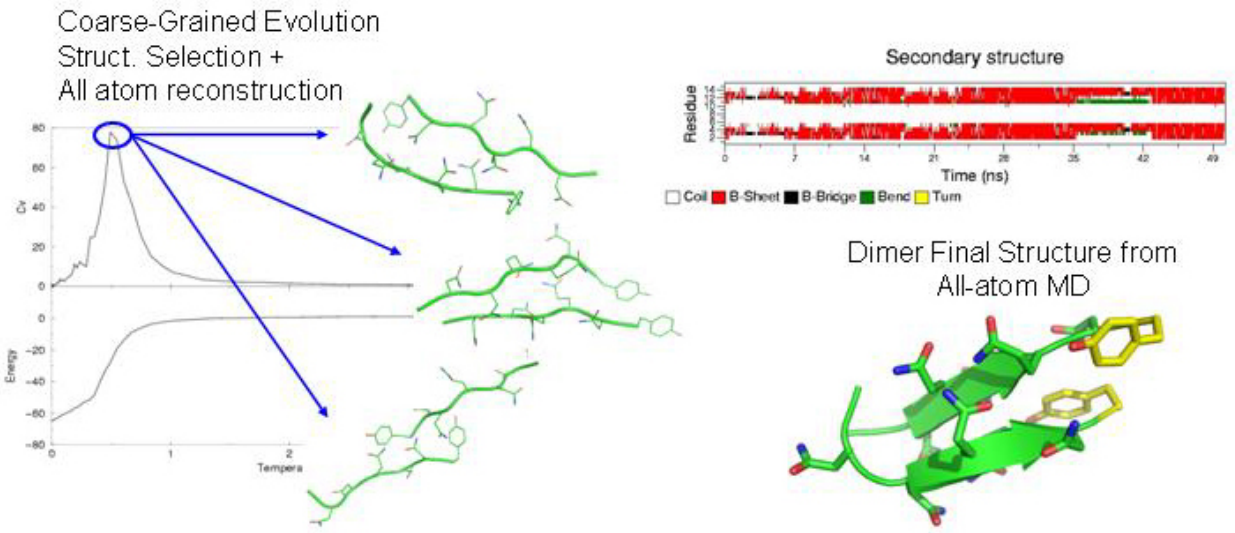

Figure 3. Schematic of the coarse-grained evolution and structure selection, combined with all-atom MD evolution of one selected conformation of the GNNQQNY amyloidogenic peptide.

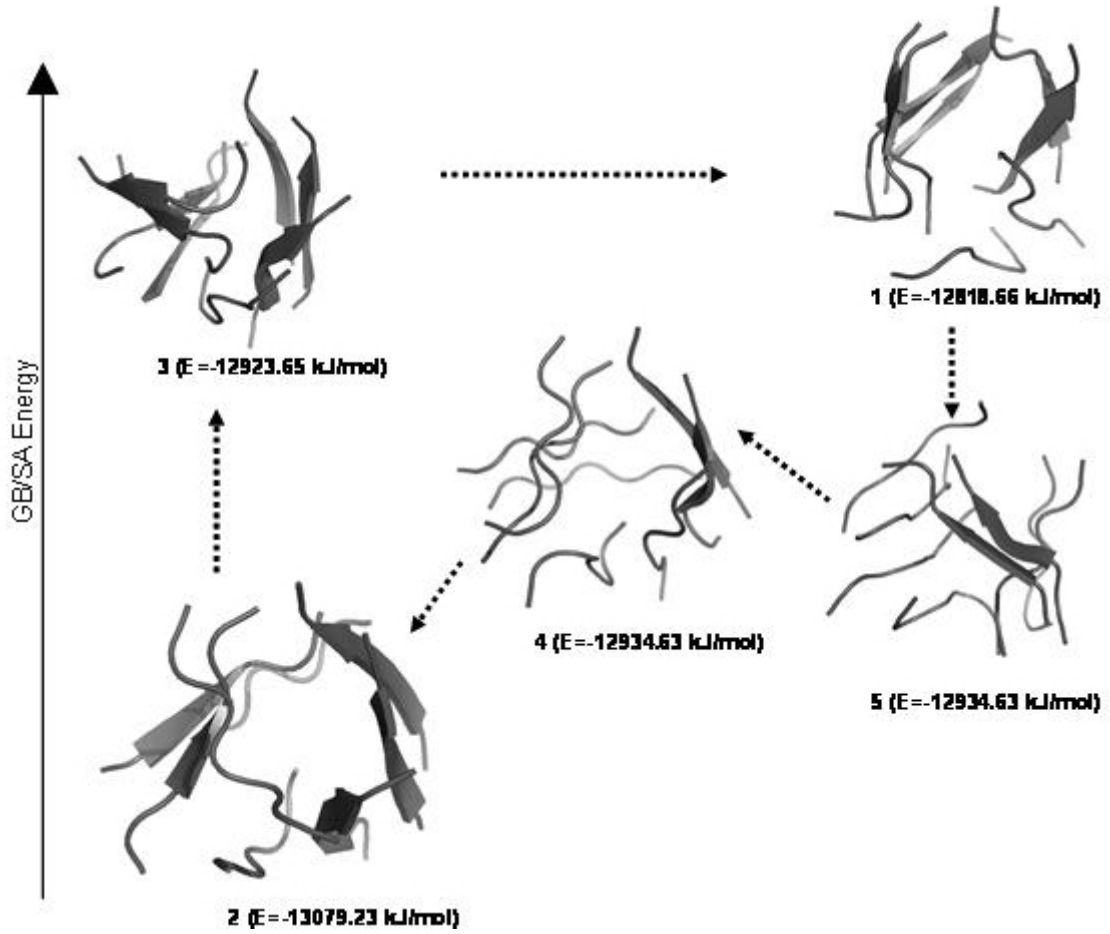

Figure 4. Structures and relative energies for the all-atom octamer simulations of GNNQQNY started from conformations selected from the coarse-grained approach.

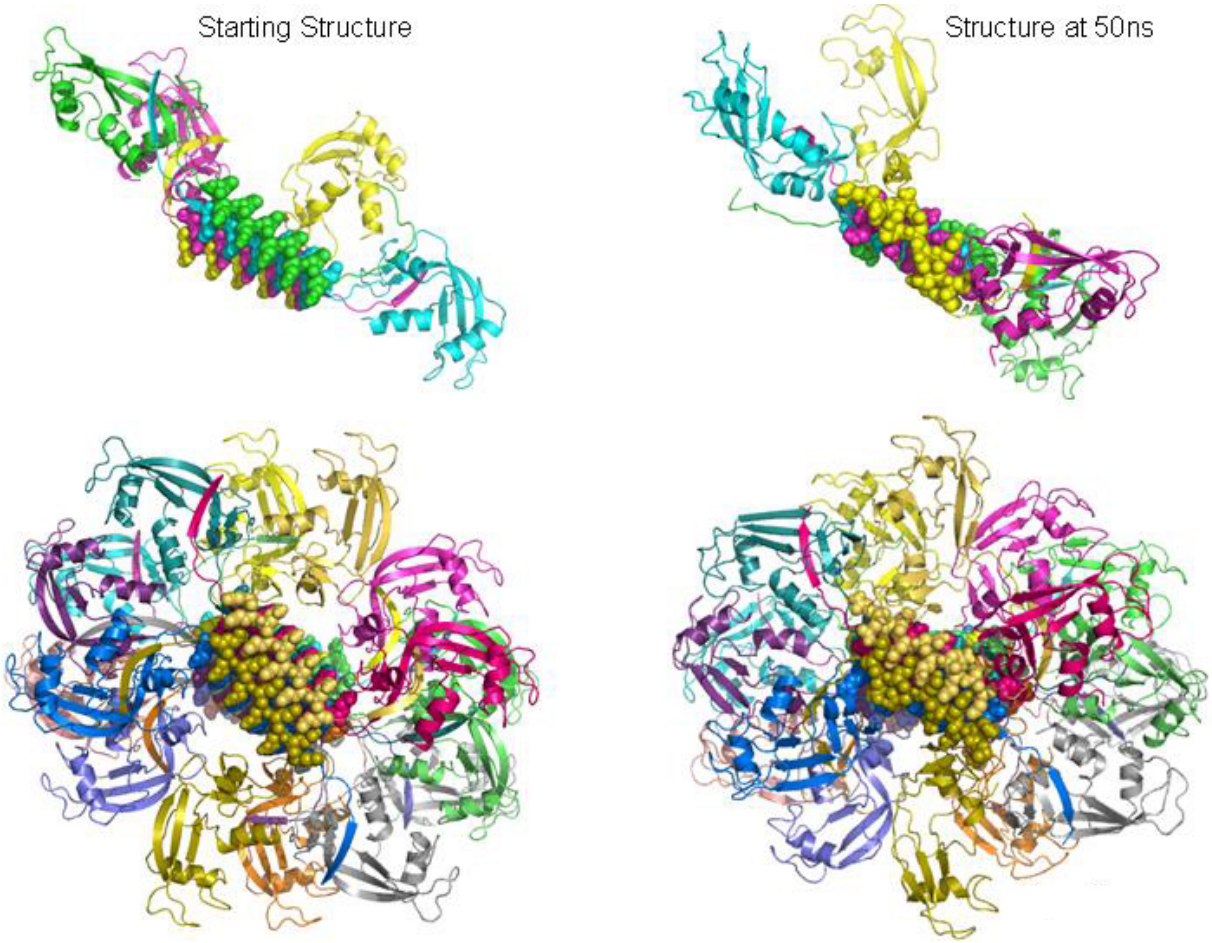

Figure 5. Starting and final structures of the RNaseA simulations with 4 and 16 chains. The structures show that the initial ideal order is maintained only in the polyQ stretch. Globular domains, while maintaining their fold, dangle in solution imparting a high degree of disorder to this region of the aggregate.

The protocol just described is as general and unbiased as possible and shows the potential to shed light on different possible organization mechanisms. Therefore, the proposed strategy might be applicable to other instances of short proteins with significant advantages over the use of only MD simulations.

5. MD INVESTIGATION OF FIBRILS CONTAINING NATIVE-LIKE STRUCTURES

Despite great progress from the theoretical and experimental points of view, major questions on the characteristics of amyloid structure remain still insolute. One of the unresolved issues concerns whether native-like domains are retained or are refolded in the fibrils. This problem is relevant, for instance, in the study of microglobulin aggregation. In that case, Fogolari and coworkers performed a 5-ns molecular dynamics simulation of an ensemble of 27 copies of beta (2)-microglobulin in explicit solvent. During the simulation, the formation of intermolecular contacts was observed and the simulation highlighted the importance of apical residues and, in particular, of those at the N-terminus end of the molecule. The most frequently found pattern of interaction involved a head-to-head contact arrangement of molecules. Hydrophobic contacts appear to be important for the establishment of long-lived (on the simulation timescale) contacts. These simulation results, in spite of their limited time scales and dimensions, were consistent with experimental data and in general with a parallel arrangement of intermolecular beta-strand pairs, shedding light on whole proteins aggregation pathways (69).

The question of the involvement of whole domains in fibrillization was addressed experimentally by Eisenberg and coworkers with the protein RNase A (70). Three properties of this molecule make it a convenient model to tackle the problem. First, it is tightly cross-linked by four disulphide bonds, severely restricting conformational change. Second, RNase A undergoes a 3D domain swap where it exchanges its carboxy-terminal β-strand with that of an identical molecule. From the structure of the C-terminal swapped RNase A dimer, it has been speculated (71) that if the hinge loop (residues 112−115) connecting the core domain (residues 1−111) with the

swapped domain (residues 116−124) were expanded by insertion of an amyloidogenic segment, then a domain-swapped amyloid-like fibril might form. Third, one of the two catalytic His residues of RNase A (at position 12) is on one core domain of the model, whereas the other (at position 119) is on the swapped domain from the other monomer, yielding the possibility to reform an enzymatically active, complementary dimer. With these considerations in mind, Sambashivan and coworkers engineered an amyloid fibril of RNAse A starting from the structure of the C-terminal domain swapped RNAse A dimer, expanding with an amyloidogenic segment ($GQ_{10}G$) the hinge loop (residues 112-115) connecting the core domain (residues 1-111) with the swapped domain (residues 116-124). In this model, the C-terminal strand of each RNAse A monomer breaks its non covalent connections to the core domain, exposing the Q_{10} segment, and the C-terminal strand of another monomer swaps in to take its place. The Q_{10} stretches from two domain-swapped monomers form an antiparallel β-sheet, which is then complemented by an analogous antiparallel β-sheet formed by two more domain-swapped monomers (70). Self-recognition of proteins is completed by precise complementation of the Q_{10} side chains stacking in the so-called *zipper spine* motif (Figure 5). The amyloid fibril obtained experimentally appears to be still catalytically active, suggesting that native like structures and placement of the catalytic residues have to be conserved to some extent in the aggregate state. Diffraction experiments on the aggregates show only the 4.8 and ~10 A reflections typical of amyloid fibrils and consistent with contributes only from the spine (Figure 5). No ordered reflection that can be ascribed to the globular domains appear to be present.

This RNAse A amyloid fibril represents an ideal and challenging test system for the MD investigation of different properties such as the stability, dynamics and conservation of native-like properties of the globular domains in the fibril model. In order to achieve these goals, we carried out explicit water MD simulations of several models of different sizes of the designed RNase A fibrils. In particular, the different fibril models analyzed consisted of 2, 4, 8 and 16 chains.

The results of our 50ns long simulations showed that the geometry of the reconstituted catalytic machinery is always conserved, and that the residues belonging to the active site always display a minimal flexibility when compared to other regions of the oligomeric assemblies. The analysis of supramolecular and collective properties of the constructs showed interesting features, highlighting the difference in dynamics connected to the increase in the number of monomers in the fibril. In the dimeric construct, in particular, the steric zipper region displays a high flexibility. In contrast, the flexibility of the steric zipper region appears to decrease as the fibril's dimensions increase. Constructs of increasing dimensions can be stabilized by the increase in the number of attractive interactions (h-bonds and van der Waals contacts) present in the central amyloid β-spine formed by the Q_{10} expansions. The interactions that stabilize the polyQ steric zipper do not perturb the folding of the remaining globular domains. The globular domains appear to protrude from the stable spine of the fibril and dangle in solution without stabilizing in one single global minimum structure. During the simulations, the positioning and orientation of the globular domains around the spine is different from the initial positions obtained from the initial structure provided by Eisenberg's lab. The globular domains take up no ordered positions around the spine, so that one would not expect diffraction from them to be coherent in X-ray experiments, and to display distinct reflections. These observations help explain why only the 4.8 and ~10 A reflections of the spine are actually observed experimentally.

The calculation of the collective properties of the four fibril models showed concerted clockwise twisting of two globular domains involved in domain swapping with each-other (Figure 5). Observation along the cross-section of the protofibril suggests that this motion will impose a left-handed twisting on the fibril, consistent with previous observations on the spontaneous twisting of model protofibrils of smaller peptides (72).

Finally, the use of an explicit water solvent model allowed us to investigate the role of water-Gln interaction in single sheet models and to hypothesize a possible role for this interaction in the mechanism of protofibril formation. Our analysis showed that polyglutamine stretches of the domain-swapped ribonuclease tend to minimize the interaction with water in favor of sidechain-sidechain interactions, supporting the idea that the pathway for amyloid fiber formation proceeds first through the formation of "dewetted" single sheets, followed by a zippering of two-sheets into a spine. Hydration analysis also showed that the patterns of hydration necessary to preserve the right active site conformation of His12 and His119 in the domain swapped arrangement are conserved, helping explain the conservation of enzymatic activity in the full fibrils.

This study represents the first case in which the properties of a complex, supramolecular aggregate from a whole protein were analyzed with atomistic methods (73). The extension of this approach to other constructs of different proteins may provide important and general information on the determinants of self-organization of biological systems, and may be used to generate ideas for the design and modification of supramolecular protein based systems useful in biotechnological applications.

6. CONCLUSIONS

The examples and the papers described in this review show that simulations have reached a stage of their evolution where provide evidence for the influence of specific interactions with well-defined stereochemical constraints on fibril formation. In the field of aggregation, in particular, MD simulations are helpful in yielding access to atomic-details underlying the stabilization of supramolecular species which are otherwise inaccessible with other types of approaches. The results from our and

other groups suggest that simulations can actually be applied to detect the critical physicochemical determinants of a certain process and can be helpful in the design of new chemical systems and experiments.

Improvement in simulation algorithms and in computer power are likely to allow researchers to access longer timescales than the ones accessible at the moment. In particular, the combination of different levels of representation using a multiscale modeling approach (coarse-grained and all-atom) is likely to advance our understanding of the mechanisms and structures of oligomers responsible for the toxicity of peptide aggregates.

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Abbreviations: MD: Molecular Dynamics; REMD: replica exchange molecular simulations; MC: Monte Carlo.

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