Theoretical Approaches to Protein Aggregation

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Abstract: The process of protein misfolding and aggregation has been associated with an increasing number of pathological conditions that include Alzheimer's and Parkinson's diseases, and type II diabetes. In addition, the discovery that proteins unrelated to any known disorder can be converted into aggregates of morphologies similar to those found in diseased tissue has lead to the recognition that this type of assemblies represents a generic state of polypeptide chains. Therefore, despite the enormous complexity of the *in vivo* mechanisms that have evolved in living organisms to prevent and control the formation of protein aggregates, the process of aggregation itself appears ultimately to be caused by intrinsic properties of polypeptide chains, in particular by the tendency of the backbone to form hydrogen bonds, and be modulated by the presence of specific patterns of hydrophobic and charged residues. Theoreticians have just recently started to respond to the challenge of identifying the determinants of the aggregation process. In this review, we provide an account of the theoretical results obtained so far.

Keywords: Protein misfolding, protein aggregation, amyloid fibrils, molecular dynamics, sensitive regions for aggregation, aggregation propensity, aggregation mechanism.

1. INTRODUCTION

The manner in which peptides and proteins operate in the dense cellular environment is subject to a complex network of strict regulatory mechanisms that have evolved to supervise folding, avoid aggregation, activate post-translational modifications, direct transport and initialise proteolysis [1]. Increasing evidence indicates that failures in any of these mechanisms can result in the formation of potentially harmful deposits that often contain amyloid fibrils [1, 2]. About 20 diseases, including several neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, and other pathologies such as type II diabetes, have been linked to the presence of this type of aggregates [3, 4].

Although initially described in association with medical conditions, it has been found that proteins not associated with known diseases can form assemblies that exhibit the characteristic cross-β diffraction pattern of amyloid fibrils [5]. The formation of aggregates with similar structural features by numerous proteins and peptides unrelated in sequence, structure and function suggests that a generic mechanism governing the process of amyloid formation may exist [6], despite the several specific features that characterise the *in vivo* aggregation of each particular protein or peptide, which include the effects of macromolecular crowding [7], the interaction with molecular chaperones [8] and proteases [9]. Given the evidence available to us at present, amyloid aggregates appear therefore to be a generic state of polypeptide chains. Indeed, the formation of this type of assemblies has been recognised as a major problem in biotechnology, which can make the process of expression and purification of recombinant proteins extremely laborious [10, 11]. In addition, specific examples have now been found of the functional use of amyloid aggregates in living organisms [12], and their exploitation in nanotechnology is being investigated [13, 14].

In this review we present a perspective on the specific contributions provided by theoretical approaches to identify the principles governing the process of ordered aggregation of peptides and proteins.

2. MOLECULAR SPECIES THAT INITIATE THE AGGREGATION PROCESS OF GLOBULAR PROTEINS

It has been suggested that the ordered aggregation of globular proteins requires the partial unfolding of the native state into an amyloidogenic intermediate state, which is populated only transiently and that exposes aggregation-prone regions normally buried within the native structure [15-17]. For example, two naturally occurring mutational variants of human lysozyme that exhibit an enhanced propensity to aggregate were shown to have a decreased stability with respect to the wild type protein and to populate a partially unfolded state [18]. Other mechanisms, however, are also possible and experimental studies have suggested that aggregation may also start from the denatured state [19], or from the native state if locally disordered regions are present [20], or perhaps through domain swapping [21].

Computer simulations of the aggregation process of proteins that use structural models of low resolution have been carried out to investigate the competition between folding and aggregation. According to several of these studies, aggregation does not seem to occur from the random coil phase but rather from intermediates populated during the folding process [22-24]. The extent to which these intermediates are native-like may depend on the type of secondary structure present in the native state. Aggregation-prone intermediates

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of proteins in the all-β class were shown to contain considerable amount of native structure, at least with respect of proteins in the all-α class [25]. Broglia *et al*. proposed that partially folded intermediates can control both the folding and aggregation process, having carried our simulations in which residues forming contacts in the aggregation-prone intermediates eventually built up the folding nucleus [26]. However, at least for small proteins and peptides, other computational studies indicated that aggregation can also take place from the unfolded state directly [27-29]. In particular, ordered aggregation and fibril formation was only observed when little residual native structure is present, i.e., for temperatures higher than the melting point (T_m) [28, 29].

Hence, the evidence provided so far by theoretical studies suggest that the aggregation process can arise mainly from partially folded conformations; whether complete unfolding is required may depend on the specific polypeptide sequence and on the external conditions.

3. MECHANISMS OF AGGREGATION

One of the first models proposed to account for the self-replication of the conformational changes associated with the formation of the amyloidogenic intermediate state, known as the "templated assembly" model (TA) [30], suggested that the aggregated state can act as a template for the further attachment of soluble monomers in their amyloidogenic form. An alternative explanation was later provided by the "nucleation-polymerisation" model (NP) [31], which assumes that the rate-limiting step, also known as "lag phase", is the formation of an oligomer sufficiently large to be stable (nucleus) above a critical concentration. After this event has taken place, the growth of the aggregate proceeds by further addition of monomers. More recently, the "nucleated conformational conversion" (NCC) mechanism was proposed for ordered aggregation, by incorporating elements of the TA and NP models [32, 33]. In the NCC model, ordered nuclei emerge due to rearrangements of amorphous oligomeric intermediates that are formed by partially or completely unstructured polypeptides. Once the nuclei are present, the growth process proceeds rapidly via a templating mechanism.

In agreement with all these models, coarse grained simulations suggested that it is kinetically easier to add a monomer to a preformed (n)-oligomer than forming the (n+1)-oligomer spontaneously [23, 27]. However, in order to distinguish clearly the TA, the NP and the NCC models, the lag time of fibril formation and the fibril growth rate have to be monitored as a function of the monomer concentration. In a recent study, Nguyen and Hall investigated the concentration dependence of the lag time [28]. At high temperatures, they observed an exponential decrease of the lag time as a function of monomer concentration, a result in agreement with the predictions drawn from the NP model. Instead, at low temperatures they found a linear dependence more compatible with the predictions of the TA model. Moreover, as also found in other simulation studies [28, 34-37], the formation of amorphous aggregates preceded β-sheet formation, a finding consistent with the NCC model. Therefore, by considering existing computational studies, none among the TA, NP and NCC models provides a comprehensive description of the aggregation kinetics observed experimentally.

4. ENVIRONMENTAL FACTORS PROMOTING AGGREGATION

The formation of amyloid aggregates depends strongly on experimental conditions, including temperature, ionic strength, pH and peptide or protein concentration [38]. Theoretical studies are now starting to appear where these factors have been considered, and some general tendencies have been reported [27, 39].

The formation of ordered aggregates can only be observed in computer simulations within a certain polypeptide concentration range. Within this range, oligmerization is initiated by the formation of disordered aggregates that eventually transform in ordered β-sheets and fibrils [28, 35, 36]. At lower concentrations, non-interacting monomers are most commonly observed. At high concentrations, disordered aggregates are formed that do not convert into order forms for long times; in the language of the physics of amorphous systems they tend to exhibit glassy behaviour [27]. The structures of the monomeric and oligomeric states in these different phases and the localisation of the phase boundaries are expected to change as a function of external factors, such as temperature, pH and ionic strength. Of particular interest is the effect of temperature on the assembly process in the concentration range where ordered aggregation can be observed. Temperatures close to the melting temperature T_m [27, 40] or slightly above it [28, 40] have been found to be particularly suitable to promote ordered aggregation. Major increases ($T>>T_m$) or decreases ($T<T_m$) in the temperature reduce the probability to observe ordered aggregates; at high temperatures, aggregates are unstable and at low temperatures peptides and proteins remain trapped in amorphous aggregates [28, 29, 34, 40, 41]. The results of several computer simulations studies of low resolution models indicated that the boundary between the ordered and amorphous phase also depends on the type and strength of the attraction between the interacting monomers [42-44]. In particular, if strong non-native hydrophobic interactions govern the assembly process, disordered aggregates are observed [42]. Moreover, by increasing the strength of interactions between non-polar side chains relative to the strength of hydrogen bonds, amorphous rather than fibrillar aggregates were observed [28]. These results support the suggestion that the protein aggregation process depends on the physico-chemical features of the amino acids forming the sequence of the protein [45] in addition to environmental factors [46].

5. DRIVING FORCES FOR AGGREGATION

Computer simulations carried out using models with different degrees of structural resolution clearly indicate that hydrophobic effects play a crucial role in promoting the aggregation process [37, 47, 48]. Polypeptide chains undergo a hydrophobic collapse that minimises the solvent accessible surface area of the protein and creates compact conformations; to compensate partially for the loss of conformational entropy associated with the structural compaction, backbone hydrogen bonds are formed concomitantly [47]. These early collapsed assemblies appear to be mainly amorphous or only partially ordered [28, 35, 37, 47]. Depending on the conditions chosen in the simulations, e.g. temperature or density,

these types of aggregates can eventually be converted into ordered ones. The simulation of the aggregation process of three short peptides from the N-terminal domain of the yeast protein Sup35 showed that the amorphous aggregates are characterised by less favorable inter-molecular interaction than the ordered β-sheet assemblies [35]. Taken together, the results of these studies suggest that amorphous aggregates do not occur because they are more stable than ordered β-sheet aggregates, but rather because they are readily accessible, i.e their formation appears to be kinetically driven [47]. The transition to ordered aggregates [28, 35, 37] requires the rearrangement or even the dissociation of the monomers in the transient disordered assembly, and the driving force for these changes appears to be mainly the optimisation of the inter-molecular interactions of the backbone as well as of the side-chains, as the transition from disorder to order is driven by an increased number of favourable interaction in the ordered form [35, 48]. Accurate molecular dynamics simulations of the aggregation process of systems composed by a small number of peptides indicate that backbone hydrogen bonds, as well as hydrophobic (in particular stacking of aromatic groups) and electrostatic (salt bridges) side-chain interactions stabilise the ordered β-sheet aggregates [35, 48]. Moreover, simulations carried out to test the stability of oligomers prepared in different conformations of high symmetry indicated that a coherent organisation of hydrophobic and polar residues in the space between β-sheets is crucial for the stability of the ordered aggregates [49, 50]. Different alignments of the monomers - parallel or antiparallel, in-register or out-of-register - have been observed in this type of computer simulations. Among these different alignments, the best interaction patterns were shown to depend on the polypeptide sequence and on its length [35, 49, 51]. If the assumption is made that fibrils are structures that maximise backbone and side-chain interactions (see below), the ordered aggregates with the lowest energies found in these calculations might represent the building blocks of the corresponding amyloid fibrils. It was shown recently, however, that also partially ordered aggregates can have a very long relaxation time [47] and it was suggested that partially ordered assemblies might participate in larger oligomers formation before relaxation takes place, i.e., they may convert in a fully ordered form [47].

6. TOWARDS AN ENERGY LANDSCAPE FOR AGGREGATION

The picture emerging from theoretical studies of the equilibrium and kinetic behaviour of peptides and proteins at high concentrations suggest the existence of a much more complex energy landscape for aggregation (Fig. **1**) relative to that for folding [52-54]. Small globular proteins appear to be characterised by a funnel-like landscape that illustrates their ability to reach their functional states rapidly and reliably. The funnel shape of the protein folding landscape arises from an evolutionary process that results in the selection of polypeptide chains for which native contacts are on average more favourable than non native ones [55, 56]. At high concentrations, in contrast, native contacts enter in competition with a vast number of alternative inter-molecular interactions that increase the ruggedness of the protein aggregation landscape.

The region of the energy landscape for aggregation that correspond to high energy and high entropy represents a situation in which soluble monomers and a range of small oligomers of different sizes are present in solution. These species are highly dynamic and interconvert rapidly to sample a heterogeneous ensemble of conformations with different amounts of native and non-native, as well as intra-molecular and inter-molecular contacts [38, 57]. The region characterised by low energy and low entropy is instead partitioned into three distinct types of assemblies: (i) crystal structures, which are stabilised mainly by the formation of intra-molecular interactions; (ii) ordered aggregates (amyloid fibrils), in which inter-molecular interactions are more predominant; (iii) amorphous aggregates, which are characterised by an irregular packing of the polypeptide chains. The

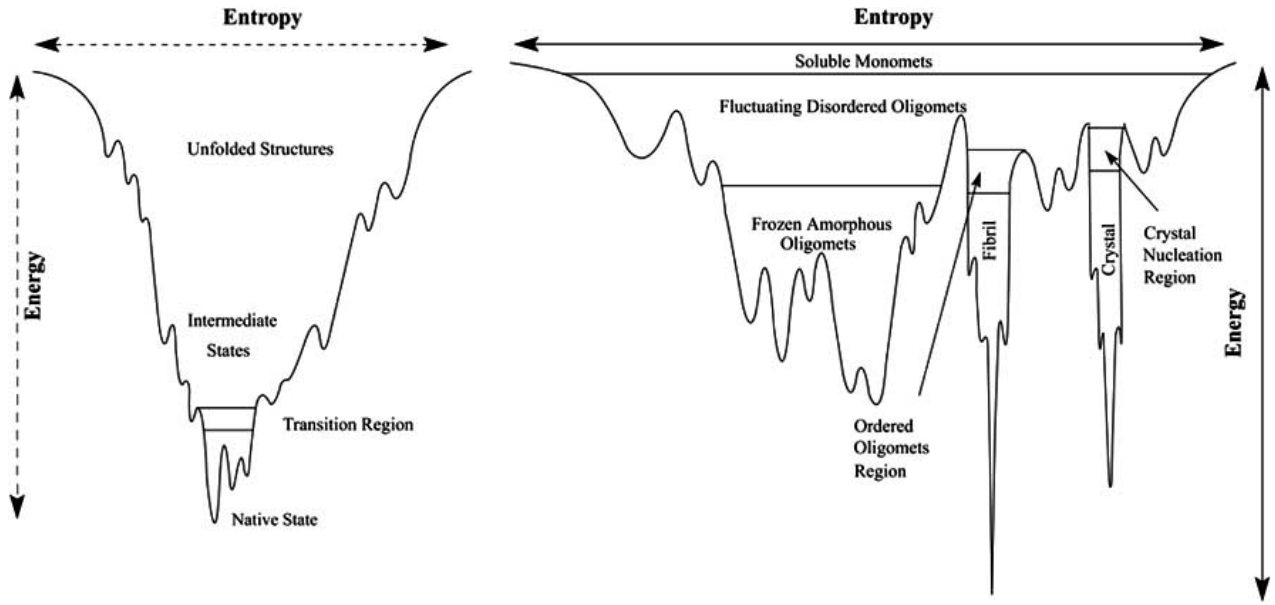

Figure 1. Illustrations of the energy landscapes for protein folding (left) and protein aggregation (right).

establishment of ordered species, either crystals or amyloid fibrils, is possible if the environmental conditions are such that the internal dynamics of the "fluctuating disordered oligomers" (see Fig. **1**) is faster than their coalescence time. In this case the lifetime of the species at intermediate values of energy and entropy (the "crystal nucleation region" and the "ordered oligomers region" in the protein aggregation landscape) is long enough to enable a nucleation process to take place.

7. STRUCTURAL MODELS OF AMYLOID FIBRILS

It has been difficult to obtain high resolution structures of amyloid fibrils, as these species are insoluble and non-crystalline. Recent advances in experimental methods in structural biology, however, are starting to provide detailed pictures of their architecture [58-63,68,97-99]. Solid-state NMR studies have resulted in direct measurements of interatomic distances in the amyloid fibrils formed by the Aβ peptides [62] and by an 11-residue fragment, denoted as $TTR_{105-115}$, of the protein transthyretin [58, 59, 64]. The experimental data on the Aβ peptides indicated that the arrangement of the β strands in the fibrils depends, among other factors, on the length of the peptide. For the $A\beta_{10-35}$ and $A\beta_{1-40}$ peptides, an in-register parallel organisation of the strands in the β-sheets was proposed [62, 65]. In contrast, the $A\beta_{16-22}$ and $A\beta_{34-42}$ were shown to form antiparallel β-sheets [66, 67]. Simulations of atomistic models of the different Aβ peptides, probing either the stabilities of various structural arrangements of octamers [50] or the aggregation mechanism of dimers [51], found the most stable and lowest energy structures, respectively, in best agreement with these experimental data. As these simulations are unlikely to cover the structural complexity present in the fibrils and the timescale of amyloid formation, their consistency with the experimental data is intriguing and suggests that polypeptide chains adopt structures in fibrils that maximise intermolecular interactions. Ma and Nussinov proposed a bended parallel β-sheet for Aβ in order to get the highest amount of hydrophobic interactions and allow for the formation of a salt bridge [50]. Independently, similar models were proposed for the structure of Aβ in a fibril based on solid-state NMR data [62,100]. In molecular dynamics simulations of the aggregation process of the GNNQQNY peptide from the N-terminal domain of the yeast protein Sup35, an in-register parallel arrangement of the β-strands resulted in the largest number of stacked aromatic groups and side-chain hydrogen bonds [35]. The same alignment and interaction pattern was observed in the X-ray structure very recently determined of amyloid microcrystals formed by Sup35 [68].

As mentioned previously, the particular alignment that provides the best interaction pattern between monomers depends on the polypeptide sequence and length [62]. Moreover, simulations of dimer [51] and trimer [35] formation indicate that for given polypeptide sequences several minima with different β-strand orientations and type of register exist besides a ground state aggregate with the largest number of favourable interactions [51]. Therefore it was proposed that the alignment giving the highest number of favourable interactions may change under different environmental conditions. In this context it is interesting to note that recent experiments showed that different Aβ fibrils, grown under either quiescent or agitated experimental conditions, appear to have different molecular structures [69]. Moreover, Sup35 amyloids formed at different temperatures were shown to adopt distinct, stably propagating conformations [70].

8. COMPETITION BETWEEN FOLDING AND AGGREGATION IN GLOBULAR PROTEINS

The sequence of most globular proteins have evolved to enable them to independently reach their functional state *in vivo* by avoiding misfolding [1]. It was estimated that, for example, in *E. coli* only about 5-10% of all proteins employ molecular chaperones to assist folding [71].

One of the strategies adopted by proteins to promote folding involves the inclusion in their amino acid sequences of motifs that help preventing aggregation. Bioinformatics approaches have been used to identify such motifs in folded proteins. Richardson and Richardson found that edge-to-edge aggregation can easily occur for protein in the all-β class, unless the edge strands use negative design [72]. As a high amount of dangling hydrogen bonds promotes edge-to-edge association, proteins reduce the number of unsatisfied hydrogen bonds or protect free edges with large loops. As another strategy to prevent aggregation, proteins utilise the placement of an inward-pointing charged residue on the hydrophobic side of a β-strand. The presence of a charged residue prevents aggregation because of electrostatic repulsion or the need to solvate this residue.

The type of design principles discussed above are effective in preventing fully folded proteins from aggregation. Other strategies have been developed by polypeptide chains to avoid aggregation during the early stages of the folding process. A coarse-grained study investigating the effect of different interatomic interaction potentials on the folding and aggregation propensity showed that at high interaction strength sequences have a high probability to have a compact native state but also to form aggregates [44]. Further, this study indicated that at very low interaction scales, non-compact structures may become the native state of a sequence, and that the number of sequences that have compact native states and are soluble is maximal for a fine-tuned value of the average interaction potential. Interestingly, protein sequences selected by such a fine-tuned potential were found to have a well-defined ratio of hydrophobic and polar residues similar to the one observed in naturally occurring proteins. Hence, the presence of an excessive number of hydrophobic residues in the sequence of a protein might increase its risk of aggregation. Indeed, a survey of the Protein Data Bank [73] suggested that proteins have evolved to avoid long stretches of hydrophobic amino acids. These results are also in agreement with the computational studies discussed above that indicated that hydrophobic interactions are important in the early stages of the aggregation process.

9. SEQUENCE-BASED PREDICTION OF AGGREGATION PROPENSITIES

In a recent seminal study, a correlation was observed among the change in the aggregation propensity of AcP upon amino acid substitution and three biophysical properties of the sequence, hydrophobicity, charge and secondary structure propensity [74]. These factors were included in a for-

mula to predict the change in aggregation rate upon the substitution of amino acids in the regions of the sequence of a protein most important for determining its aggregation propensities [45]. This observation spawned a series of successive studies. Tartaglia *et al.* [75] showed that the surface exposed area of unstructured peptides, the dipole moment of side-chains and π-stacking propensities of aromatic residues can be used in addition to the factors already identified by Chiti *et al.* [45] to predict the effect of single residue mutations on the aggregation rates. In another study, the formula of Chiti *et al.* [45] was extended to predict the absolute aggregation rates of polypeptide sequences [46]. In this case the experimental conditions were taken into account into a formula, applicable to unstructured peptides and natively unfolded proteins, capable of predicting the aggregation rates of polypeptide chains over a range spanning five orders of magnitude [46]. More recently the same approach has been applied to identify the sensitive regions for aggregation [76]. In an application to three polypeptide chains involved in neurodegenerative diseases (Aβ, α-synuclein and tau) it was shown that there are two types of sensitive regions; aggregation-prone regions, which have a high intrinsic tendency to aggregate and aggregation-susceptible regions, which may become sensitive if certain amino acid substitutions occur, either naturally or by design. Sensitive regions for β-sheet aggregation were identified by using similar principles by Serrano and coworkers [77-79]. They proposed an algorithm that, in addition to the factors identified by Chiti *et al.* [45], considers also the competition between β-sheet formation and other structured states of the sensitive regions, and includes the change in stability of the native state of folded proteins upon mutation [77, 78] using the program fold-X [80]. The inclusion of the stability is an important step, since the intrinsic aggregation propensity of a given sequence is expected to be modulated by the exposure to the solvent in folded proteins or partially structured intermediates [15-17].

10. STRUCTURE-BASED PREDICTIONS OF AGGREGATION PROPENSITIES

The combination of rapid folding and the intervention of molecular chaperones appear to be able to prevent the aggregation of proteins containing in their sequence regions with a high propensity for aggregation [8]. However, structural changes in the native state, such as those induced by mutations or by changes in the environmental conditions (e.g. pH, temperature), can impair the capacity of proteins to remain folded [6, 18, 81]. A number of molecular dynamics simulations of folded proteins have been carried out in recent years to investigate the effects of low pH and mutations on the structure of amyloidogenic proteins [82-85]. As the simulations of these complex models are limited to the nanosecond timescale, they can only allow a glimpse into the initial events of the conformational transitions to the aggregation-prone form. Most interestingly, recent simulations of transthyretin, β2-microglobulin, lysozyme and the prion protein at low pH showed the formation of α-pleated sheet structures [83, 86]. The authors suggested that this type of structures may represent a common conformational transition step in the process of fibril formation.

Several bioinformatics studies have been presented that identify structural defects leading to aggregation by comparing the structures of amyloidogenic and non-amyloidogenic proteins. In particular, it was investigated whether defects promoting aggregation may be found in secondary structural elements or in the sequence as a whole [27, 87]. It was proposed that the degree of frustration of secondary structure elements can report on the amyloidogenic tendencies. Secondary structure elements are said to be frustrated if their theoretical prediction differs from their experimental determination [88]. It could be shown that several amyloid-forming proteins harbour α-helices in regions that are theoretically predicted to be β-strands [27, 87]. In agreement with the work of Richardson and Richardson mentioned above [72], it was proposed by Fernandez and coworkers that structural defects leading to aggregation might also originate from an insufficient desolvation of amide carbonyl hydrogen bonds [89].

11. AVOIDANCE OF AGGREGATION BY NATIVELY-UNFOLDED PROTEINS

Natively unfolded proteins lack persistent secondary and tertiary structure under physiological conditions, at least in the absence of binding partners [90], and therefore they represent an intriguing case from the point of view of aggregation, as they seem to populate naturally a state with a high amyloidogenic potential. It seems clear therefore that strategies should have evolved to neutralise *in vivo* their intrinsic propensity to aggregate. Uversky and Fink suggested that the sequences of natively unfolded proteins are characterised by a different distribution of hydrophobic and charged residues [17]. Further, the study of Linding *et al.* [78] mentioned above suggested that the sequences of natively unfolded proteins contain on average only one third of the aggregation-prone regions relative to globular proteins. Since in globular proteins, however, most of the aggregation-prone regions are buried within the folded structure, the absolute tendency to aggregate is similar for natively unfolded and for globular proteins. More recently, a study by Pawar *et al.* [76] indicated that it is possible to define an intrinsic amino acid scale for aggregation. In this scale, hydrophobic amino acids exhibit a high tendency to promote aggregation; charged ones tend instead to reduce such a tendency. The strategies for avoiding aggregation and folding appear therefore to be based on similar principles. Indeed, the amino-acid propensities for being intrinsically unfolded [90] and the propensities for aggregation [76] are anti-correlated (Fig. **2**). These observations are in agreement with the study by Giugliarelli *et al.* [44], indicating that sequences which have compact native states also form aggregates, in contrast to those having non compact structures as their native state.

In addition to these general considerations, the development of computational methods capable of determining accurately the structural preferences in partially folded states of proteins [91] is making it possible to obtain a more detailed understanding of the mechanisms by which natively unfolded proteins are capable of avoiding aggregation. A recent structural study of α-synuclein [92], a natively unfolded protein abundant in the brain that has been shown to be a major component of the Lewy bodies observed in the pathogenesis of Parkinson's disease, revealed that the highly charged C-terminal region has an enhanced tendency to fold back on to the central NAC region of the sequence, which is

thought to be aggregation-prone [93], and thus partially prevent it from forming inter-molecular interactions. These results suggest that even weak conformational preferences encoded in the amino acid sequence of a protein may play an important role in determining the overall behaviour of proteins with respect to aggregation and help clarifying how the side-chains can modulate the intrinsic propensity of a polypeptide backbone to form amyloid aggregates [94].

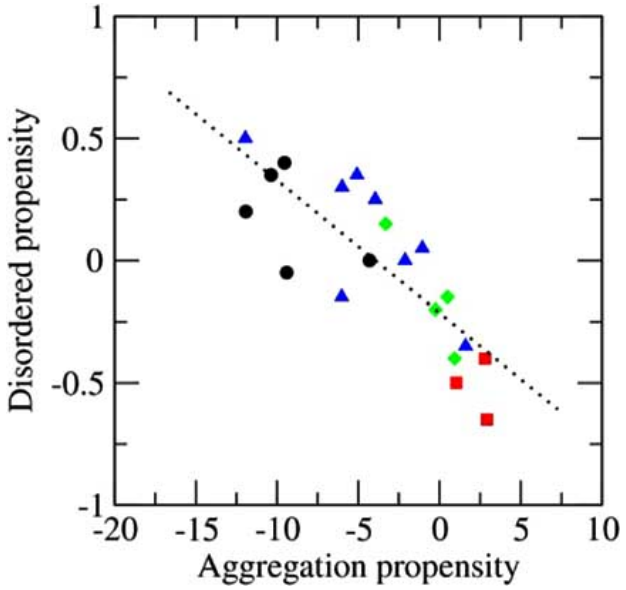

Figure 2. Comparison between the amino acid propensities for aggregation [76] and for disorder [90]; aromatic residues are shown in red, hydrophobic residues in green, polar residues in black and the remaining ones in blue.

12. CONCLUSIONS

In the last few years significant advances have been made, through a combination of experimental and theoretical studies, that are starting to provide a glimpse at the principles determining the process of protein aggregation and at the structures of the aggregated species. Although in systemic amyloidoses the deposition of large quantities of protein aggregates appear to have direct pathological consequences through the disruption of tissues [95], the main mechanisms of toxicity of protein aggregates seems to involve mainly the oligomeric species that appear transiently during the early stages of aggregation [2, 96]. Therefore the understanding of the process of protein aggregation, and the development of rational strategies to combat it, represent crucial challenges for theoreticians.

ABBREVIATIONS

NP = Nucleation-polymerisation model

TA = Templated assembly model

T_m = Melting point

NCC = Nucleated conformational conversion

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