Aggregation Prone Regions and Gatekeeping Residues in Protein Sequences[†]

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Abstract: Most protein sequences contain one or several short aggregation prone regions (APR) that can nucleate protein aggregation. Under normal conditions these APRs are protected from aggregation by protein interactions or because they are buried in the hydrophobic core of native protein domains. However, mutation, physiological stress or age-related dysregulation of protein homeostasis increases the probability that aggregation-nucleating regions become solvent exposed. Aggregation then results from the self-assembly of APRs into β -structured agglomerates that vary from small soluble oligomeric assemblies to large insoluble inclusions containing thousands of molecules. The functional effects of APR-driven aggregation are diverse and protein-specific leading to distinct disease phenotypes ranging from neurodegeneration to cancer. On a cellular and physiological level both wild type loss-of-function as well as aggregation-dependent gain-of-function effects have been shown to contribute to disease. Several molecular mechanisms have been proposed to contribute to gain-of-function activity of protein aggregates including cellular membrane dysregulation, saturation of the protein quality control machinery or the ability of aggregates to engage non-native interactions with proteins and nucleic acids. These different mechanisms will all, to some extent, contribute to gain-of-function as in essence they all contribute to the rewiring of the cellular interactome by aggregation-specific interactions, resulting for instance in the pronounced neurotoxicity of TDP43 aggregates by the sequestration of RNA molecules or the promotion of cell proliferation by the entrapment of homologous tumor suppressor proteins in p53 aggregates in cancer. In this review we discuss the mechanism of APR driven aggregation and how APRs contribute to modifying the cellular interactome by recruiting both misfolded as well as active proteins thereby inhibiting or activating specific cellular functions. Finally, we discuss the ubiquity of APRs in protein sequences and how selective pressure shaped protein sequences to minimize APR aggregation.

Keywords: Protein aggregation, beta-structure, aggregation prone region (APR), gatekeeper, amyloid.

INTRODUCTION

Protein aggregation is a process that has been recognized for a long time as heat coagulation was used to identify the proteinaceous nature of biological material since before 1838 when Berzelius first coined the term 'protein'. In 1935 Astbury determined the X-ray diffraction pattern of boiled egg white, for the first time showing that aggregated protein adopts an extended β -structured conformation [1], leading Perutz to recognize the conformational nature of aggregation in an article entitled 'Unboiling an egg', published in *Discovery* in 1940. Following Anfinsen's work on protein folding, protein aggregation was soon recognized to be a protein misfolding reaction competing with native protein folding [2-4], which is favoured by increasing protein concentration [5, 6]. In recent years, the mechanistic details of this competition are emerging in several studies where partially folded intermediates *en-route* to the native state were identified,

which alternatively to proper folding could also form non-native interactions with similar molecules to give rise to the aggregation nucleus [7, 8]. Since its discovery, protein aggregation has been associated to human disease and to inclusion body formation in the context of recombinant protein production. Both pathogenic deposits and recombinant inclusion bodies are highly enriched in one particular protein, suggesting the underlying aggregation mechanism is selective and specific [9-12]. In its most ordered form, protein aggregation gives rise to a fibrous deposit reminiscent of amylose, which have hence been called amyloid fibrils [13]. Interestingly, the nanostructures that are amyloid protein aggregates are also employed by nature, which e.g. exploits these microscopic wires for their tensile strength and adhesiveness, most famously perhaps as spider silk [14-16]. Other examples include the chorion protein, which contributes to the mechanical strength of the insect egg shell or the hydrophobins, which are used as a water repellent by filamentous fungi [17]. Last, but not least, amyloid fibrils are increasingly being researched as a source of novel biosynthetic nanomaterials [18].

PROTEIN AGGREGATION IS A SELECTIVE PROCESS DRIVEN BY APRs

A growing body of theoretical and experimental data suggests the specific nature of protein aggregation is driven

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[†]This article is published as part of a themed issue on Protein Misfolding in Conformational Disorders, Guest Edited by Cláudio M. Gomes (ITQB/UNL).

by interaction of short aggregation prone polypeptide segments with specific amino acid composition [19, 20], further termed Aggregation Prone Regions or APRs for short. These aggregation nucleating segments are usually composed of 5-15 successively placed hydrophobic amino acids of low net charge that have been shown to constitute a sufficient and necessary requirement for protein aggregation [21-24]. Indeed grafting an APR on an otherwise non-aggregating protein scaffold is sufficient to induce aggregation [24, 25]. Although most proteins possess one or several APRs within their sequence [26, 27], these are generally buried in the hydrophobic core [28] or protein interaction sites [29, 30] and are thereby protected from aggregation. Solvent exposure of these aggregation prone segments in the event of transient breathing motions or protein unfolding [31, 32] initiates intermolecular association of APRs by β -strand interaction, thereby assembling APRs into β -sheet containing protein aggregates that adopt macromolecular morphologies ranging from amorphous assemblies to highly ordered amyloid fibrils Fig. (1) [33, 34]. The intricacies of the underlying energy landscapes are being revealed by a number of experimental and theoretical approaches [35, 36]. These intermolecular interactions between APRs mediating β -sheet structure are sequence dependent, resulting in a bias towards self-association over hetero-assembly [9, 12]. For example, cross-seeding of amyloid fibril formation between islet Amyloid polypeptide (IAPP) and the Alzheimer β -peptide (A β) is very inefficient compared to self seeding of each peptide, although both the peptides share 70% sequence similarity in the aggregation nucleating cores [37]. Similarly, scrambled or reversed versions of the IAPP peptides do not cross-seed with each other or the wild type sequence, confirming position dependence beyond mere sequence composition [37].

STRUCTURE AND SEQUENCE OF APRs

The aggregation tendency of a peptide segment correlates with hydrophobicity and beta-sheet propensity and is favoured by a low net charge [38, 39]. As hydrophobicity and β -sheet propensity scales are available for all amino-acids, these biophysical correlations can be converted to sequence-specific β -aggregation prediction algorithms. The first algorithm built on these principles was the statistical thermodynamics algorithm TANGO [20] that, given the amino acid sequence of a protein, accurately locates beta-aggregation

nucleating regions. Subsequently, alternative prediction algorithms to TANGO were developed (for a historic overview, see Fig. 2), that utilize similar amino acid characteristics such as hydrophobicity, beta propensity, packing density, charge constraints or derivations and combinations thereof [40-47]. A comprehensive review of APR detection methods is beyond the scope of the current manuscript, but excellent overviews can be found elsewhere [48-53].

The underlying rationale for this way of detecting aggregation prone regions (APRs) is the hydrophobic beta-structure adopted by these sequences in the final aggregate or amyloid fibril. Recently, high resolution structural studies of amyloid fibrils derived from a number of such peptides, by both Nuclear Magnetic Resonance and microcrystal x-ray diffraction, confirmed the basic cross-beta concept [13, 54-60]. However, significant variation is observed between the different structures, in terms of side chain packing, intermolecular distance and degree of regularity [21], revealing the molecular basis of the structural polymorphism observed in aggregates [61]. The essential feature is that of so-called steric zippers, where the sidechains of independently formed beta-sheets are held to together by interdigititation and close packing [56]. Based on theoretical considerations, Eisenberg proposed that eight different structural classes of such zipper arrangements would be possible for the cross-beta spine [56], and for all but one of these we have experimentally observed cases [62]. In addition, more complex structures have also been observed, such as the amyloid fibrils of the Alzheimer beta peptide, in which each molecule contributes two β -strands, connected by a loop region to the final structure [58]. Importantly, similar structures have been observed in cellular inclusion bodies, showing that the basic pattern applies *in vivo* [63].

A second generation of APR predictors exploits this new wealth of structural information directly by employing homology modelling techniques based on the high resolution structural data [27, 64], or by combining position-specific scoring matrices with such homology modelling based scores [19] Fig. (2). These predictors tend to be more specific for the amyloid fibril structure than earlier predictors, which predicted amyloid and amorphous beta-aggregation alike [19, 65]. Last but not least, the high resolution structural information is now being exploited to design sequence specific aggregation inhibitors that are designed to cap and inhibit the aggregation potential of APRs [66, 67].

Fig. (1). Transmission Electron Microscopy images of peptides corresponding to aggregation prone regions of selected proteins display a wide range of morphologies, from highly ordered straight (A) or (B) twisted fibrils, over various degrees of order (C) to completely amorphous structures (D).

Fig. (2). Historic overview of some well known APR prediction algorithms: TANGO [20], the STVIIE sequence pattern [139], Zygggregator [40], PAGE [140], 3DProfile [27, 64], SALSA [47], Aggrescan [141], PASTA [142], Betascan [143], AmylPred [144], Waltz [19] and FoldAmyloid [145].

Prion proteins occupy a particular niche in the sequence space of amyloids: these polar sequences are highly enriched in asparagine and glutamine residues, and these residues seem to generate a balance between amyloid formation, promoted by asparagine, and intrinsic disorder, promoted by glutamine [68, 69]. The effect of these two residues is so strong that a search of the yeast genome for regions with high Q/N content lead to discovery of more than 10 novel yeast prions [70] and more recently, Toombs *et al.* demonstrated that the general principle could be turned in a high accurate prion-prediction algorithm [71].

STRUCTURAL TRIGGERS OF AGGREGATION

Using APR prediction algorithms on protein domain databases such as SCOP or on full proteome sets [26, 27] reveals that about 18% on average of the residues from globular domain folds are within aggregation prone regions, irrespective of whether the protein has an all- α , all β or mixed

α/β topology, while intrinsically disordered proteins on average only have about 8% of residues within aggregation prone regions. Whereas most protein domains contain one or more APRs only about 5% of globular domains are completely devoid of APRs whereas 40% of unstructured domains are devoid of APRs, suggesting a strong protective effect of globular structure to suppress APRs [22]. This is in line with the observation that the APRs constitute the most stable regions of the hydrophobic core of the native protein [72, 73] and with earlier work into the mechanism of protein aggregation showed that aggregation often proceeds from partially folded intermediates [39, 74], consistent with the observation that APRs are protected from the solvent under native conditions by their incorporation in the globular fold of the functional protein. This then raises the question what causes protein to adopt conformations that allow APRs to nucleate aggregation under near-native conditions. An obvious mechanism is the thermodynamic destabilisation of the native structure by genetic mutation [75], post-translation modification [76] (such as tryptophan oxidation) or loss of translational fidelity [77, 78], which can lead to an increased population of the denatured state of the protein [79]. However, more recently it became clear that aggregation does not require the polypeptide to cross the major energy barrier for unfolding, but rather that local fluctuations in structure can sufficiently expose APRs to facilitate aggregation nucleation [33]. Eg, recently the near-native but aggregation prone conformations of acyl phosphatase from *Drosophila* were mapped experimentally, revealing changes in surface charge distributions, solvent exposure of hydrophobic amino acids and alterations to the topology of the elements of secondary structure [32]. In the Anthrax Protective Antigen protein, a near-surface APR was shown to initiate the aggregation reaction [80]. Another striking example is α -Chymotrypsinogen A, where aggregation is initiated through some major structural remodelling of some near-surface APRs without affecting the overall fold [81]. Such subtle fluctuations can easily be caused by protein non-intrinsic factors which then cause misfolding and aggregation in perfectly translated polypeptides with wild type sequences. This is often observed when proteins are employed for research, therapy or industrial applications, when they are expected to withstand artificial conditions for which evolution has poorly equipped them [82]. Inside the cellular context however, it is change in the proteostatic capacity of the cell that is the key factor, *ie* changes in the balance between protein synthesis, (re)folding and degradation, which has been extensively reviewed elsewhere [83-85].

AGGREGATION-SPECIFIC INTERACTOMES AND GAIN-OF-FUNCTION

The functional consequences of protein aggregation include loss of function effect as well as gain of novel functions. Although initially gain of function activity was mostly restricted to cytotoxicity, it recently became clear with aggregates of the tumor suppressor protein p53 that the functional consequences can be much more diverse, as in the case of p53 aggregation actually promotes cellular growth [86]. The molecular mechanisms by which downstream effects of protein aggregation take place are multiple, but two main categories can be discerned: (1) Sequestration of additional

Fig. (3). Schematic overview of some of the main consequences of- and cellular strategies against protein aggregation.

cellular factors [86], which includes the overwhelming of the proteostatic machinery through depletion of chaperones [87], and (2) Disruption of the structural integrity of the cell through interaction with and perturbation of cellular membranes [88, 89] or the cytoskeleton [90, 91]. Both classes of mechanisms are not mutually exclusive and both aspects may contribute to a different extent to the functional consequences of aggregation of particular proteins. A unifying factor is the observation that it is not the mature aggregates that mediate gain of function effects but the smaller soluble aggregates also known as prefibrillar oligomers [62]. For example in Alzheimer Disease, several of these species have been identified, including dimers [92], trimers [93] and A β species with a molecular weight of 56 kDa [94] to A β -derived diffusible ligands (ADDLs) [95] and annular protofibrils [96] in potent neurotoxic fractions. Recently, the first high resolution structure of an oligomeric form of an amyloid forming peptide derived from α B crystallin was determined, revealing a beta-barrel configuration [97]. Since a single conformationally specific antibody specifically recognises the oligomeric conformation of many proteins, independent of sequence, it is tempting to speculate that a similar barrel structure will be found for many other disease-associated oligomers [98].

Once the APRs start assembling into intermolecular beta-structures, the domain in which they reside is thought to be mostly unfolded, causing the loss of its function, but other domains in the protein need not be affected by the aggregation and can retain their functional fold. This is for example the case for a designed amyloid of an RNase A protein, in which the domains that are peripheral to the amyloid spine (formed by the APR) retain their globular fold [99]. A similar scenario was proposed for amyloid fibrils of the yeast prion Ure2, which also leads to the assembly of functionally intact protein into the fibrils [100, 101]. Assembly of functional protein domains into uncontrolled aggregates presents the cell with a dramatic form of dysregulation, which was beautifully illustrated with the case of TDP43, which is

found to form toxic aggregates in the motor neurons of a large fraction of patients with Amyotrophic Lateral Sclerosis (ALS) [102-104]: in this protein, the aggregation is nucleated by APRs in its carboxyterminal domain [105], but the toxicity of these aggregates hinges on the presence of a central folded RNA-binding domain in the aggregates [106]. Since this TDP-43 RNA interaction domain has binding sites within a third of all mouse and human mRNAs, sequestration of RNAs in the aggregation is likely to be the key mechanism mediating neurotoxicity [107].

Finally, the case of p53 illustrates another form of sequestration, this time by assembly of highly similar APRs into the beta-spine of the mixed aggregate. Although wild-type p53 does not interact with p63 and p73, it was known that mutant p53 binds to p63 and p73 and inhibits their tumor suppressive functions [108, 109]. We recently demonstrated that the inactivation of p63 and p73 by mutant p53 is determined by a specific APR called p53 β in its DNA-binding domain that becomes exposed in mutant p53. Association of p53 β with the homologous sequences in WT p53, p63 and p73 results in mutant p53 induced aggregation and inactivation of both WT p53 and its paralogues [110].

CELLULAR MECHANISMS AGAINST APR AGGREGATION

Protein aggregation is deleterious for the activity of the affected protein but often also affects cellular function or an organism at a systemic level. Given the strong selective disadvantage of misfolding and aggregation [111] it could seem surprising that the vast majority of proteins in any proteome possess at least one and generally several APRs. This strongly suggests that the presence of APR is somehow associated to the function and folding of proteins and cannot therefore easily be selected against [22]. Computational analysis has shown that the molecular basis of this is the fact that the same basic forces drive protein aggregation and folding [51, 112, 113], although the proportional relevance of individual components such as hydrophobicity and hydrogen

bonding may differ between globular and fibrillar structures [114]. As a result, the only group of polypeptides with a reduced overall aggregation propensity are the intrinsically unstructured proteins (IDPs)[22].

Shortly after the publication of the first APR prediction algorithm, it was employed to study the aggregation load of 28 proteomes from different kingdoms of life, which lead to the first clear statistical evidence that evolutionary pressure to reduce protein aggregation pervades all known polypeptide sequences [115]. Later, this was confirmed in other studies to be independent of method employed [116, 117] or the proteomes studied [118-120]. The key pattern that emerged is that of so-called aggregation gatekeepers flanking APRs [115]. This term was first coined during the study of the folding mechanism of C12 [121] and S6 [122, 123] to describe residues that keep the protein on the productive folding pathway to the native state. In the context of APRs, the gatekeeper term is used to indicate amino acids that disrupt the aggregating hydrophobic stretches and keep the aggregation propensity of the local sequence in check. A major component in this effect is the repulsive effect of introducing charged residues (Arg, Lys, Asp, Glu), but among these aggregation is penalised more effectively in an entropic manner [124] with the placement of large and flexible side-chains like Arg or Lys. Finally, aggregation can be controlled using residues that are incompatible with the beta-structure of aggregates, as is the case for proline and perhaps to a lesser extent also glycine [125, 126]. Interestingly, the effect of proline is stereochemically selective as it is only effective at the N-terminal side of the APR [111].

The relevance of the statistical enrichment of gatekeeper residues at positions flanking APRs was confirmed by several independent studies. First, the selective pressure against aggregation is especially pronounced in proteins with an essential cellular function [118, 127, 128]. Second, it was established that mutations that disrupt the gatekeeper pattern occur more frequently in disease-associated mutations than in polymorphisms [119]. Third, a host of experimental studies found the gatekeeper residues to be essential determinants of aggregation behaviour of the protein of interest. Eg. gatekeeper residues have been shown to modulate *in vivo* amyloid fibril formation by the bacterial curli protein [129], gatekeepers were shown to affect the aggregation rate of the SH3 domain of the PI3K protein [120] and that of the Parkinson disease-associated mutant of alphasynuclein A51T [130] and finally, gatekeeper residues modulated the *in cellulo* aggregation of the tumor suppressor p53 [86]. Further, it was independently shown that gatekeeper residues are not necessarily required to mediate native folding, but rather, specifically prevent aggregation from the unfolded state [131] (by the mechanisms discussed above such as charge repulsion). Moreover, gatekeepers participate or at least facilitate recognition of APRs by molecular chaperones, such as Hsp70, preventing intermolecular assembly and nucleation of aggregation [132]. Interestingly, we found that gatekeeper residues of an aggregating protein overexpressed in *E.coli* affected bacterial fitness by modulating the proteostatic network in an attempt to match protein expression to the toxicity of the aggregation process [111].

The final line of support for the notion that the gatekeeper pattern is an essential feature of protein architecture, comes from the study of groups of proteins in particular danger of aggregation such as proteins having a long lifetime or which are expressed at high levels. In several independent studies of these proteins it was observed that the gatekeeper pattern is evolutionarily enhanced. Proteins at risk for aggregation can easily be identified by basic considerations about the kinetics and thermodynamics of the protein aggregation reaction:

(A) Protein aggregation is a nucleation-growth phenomenon with a lag phase during which stable nuclei need to form, followed by a rapid growth phase. Thus, in short lived proteins aggregation can be prevented by their natural elimination within the lag-phase of aggregation, but long lived proteins need to be better equipped to avoid aggregation during long times scale. Accordingly we did observe that proteins with a long biological life time show a reduction in their intrinsic aggregation propensity [72].

(B) Moreover, both the rate and the extent of aggregation is strongly increased with increasing protein concentration. As a result, a more pronounced pressure to minimize aggregation is noticed in proteins with a high expression level [133], or - even more clearly - in proteins with a high abundance [134].

(C) Last but not least, the APR model of aggregation also implies that the aggregates bury significantly less hydrophobic surface area than would be the case in a globularly folded protein, since only a short stretch of the polypeptide is involved in the actual aggregate core. The result of this is that the heat capacity (ΔC_p) of the aggregates will be lower than that of folded proteins, so the native state is destabilised more per degree of temperature than the aggregated state, or in other words: increases in temperature favour aggregation over native folding. Consistent with this, in thermophilic proteins both the gatekeeper pattern and the burial of aggregating sequences are more pronounced than in mesophilic proteins [135].

Evolutionary elimination of aggregation nucleating regions is ultimately limited by the fact that protein function is the primary selection criterion. Therefore, when aggregation prone sequences do persist, they are usually found in functionally important regions of proteins, where mutations would interfere with function, such as the antigen complementation regions of antibodies [136], or protein-protein interaction regions in general [137, 138].

CONCLUSION

In summary, it is becoming clear from the recent developments in the field that protein aggregation is triggered by short aggregation prone regions or APR, that can nucleate aggregation by assembling into intermolecular beta-structures. APRs occur with relatively high frequency in protein sequences because high hydrophobicity, high beta-sheet propensity and low net charge are architectural requirements for the proper folding of an hydrophobic core in globular protein domains but these conditions unfortunately also favor protein aggregation. In conditions where the stability of the native state is intact, the APRs are buried and

thereby inert, but as soon as they become more exposed to the solvent, aggregation may be initiated. When aggregates are formed, they have a strong negative impact on cellular fitness, by sequestration of other cellular factors, by causing overload of the proteostatic machinery, by disruption of cellular membranes or by any combination of the above. Not surprisingly, there has been strong evolutionary pressure to minimize the aggregation propensity of APRs by the placement of aggregation gatekeeper residues at the positions directly flanking the APR. These act by slowing down the aggregation reaction in case the APR becomes solvent exposed, giving refolding another chance, as well as by increasing chaperone binding to the exposed hydrophobic region. However, protein structural and functional requirements limits the evolutionary pruning of APRs, and they do persist mainly in regions that are essential for either protein stability or for protein function, such as the hydrophobic core or active sites and protein-protein interaction sites.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

The VIB Switch Laboratory was supported by grants from the Flanders Institute for Biotechnology (VIB), the University of Leuven (KUL), the Funds for Scientific Research Flanders (FWO), the Flanders Institute for Science and Technology (IWT) and the Interuniversity Attraction Pole (IAP) Network from the Science Policy of the Federal Government of Belgium (Belspo).

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