RepA-WH1 prionoid: Clues from bacteria on factors governing phase transitions in amyloidogenesis

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ABSTRACT. In bacterial plasmids, Rep proteins initiate DNA replication by undergoing a structural transformation coupled to dimer dissociation. Amyloidogenesis of the 'winged-helix' N-terminal domain of RepA (WH1) is triggered *in vitro* upon binding to plasmid-specific DNA sequences, and occurs at the bacterial nucleoid *in vivo*. Amyloid fibers are made of distorted RepA-WH1 monomers that assemble as single or double intertwined tubular protofilaments. RepA-WH1 causes in *E. coli* an amyloid proteinopathy, which is transmissible from mother to daughter cells, but not infectious, and enables conformational imprinting *in vitro* and *in vivo*; i.e. RepA-WH1 is a 'prionoid'. Microfluidics allow the assessment of the intracellular dynamics of RepA-WH1: bacterial lineages maintain two types (strains-like) of RepA-WH1 amyloids, either multiple compact cytotoxic particles or a single aggregate with the appearance of a fluidized hydrogel that it is mildly detrimental to growth. The Hsp70 chaperone DnaK governs the phase transition between both types of RepA-WH1 aggregates *in vivo*, thus modulating the vertical propagation of the prionoid. Engineering chimeras between the

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Sup35p/[*PSI*⁺] prion and RepA-WH1 generates [*REP-PSI*⁺], a synthetic prion exhibiting strong and weak phenotypic variants in yeast. These recent findings on a synthetic, self-contained bacterial prionoid illuminate central issues of protein amyloidogenesis.

KEYWORDS. amyloid proteinopathy, amyloid polymorphism, bacterial prionoid, Hsp70 chaperone, phase transitions, RepA-WH1

INTRODUCTION

Along the last few years, the field of functional amyloids, i.e., amyloid assemblies with a physiological role and no proteinopathic side effects, has attracted much attention.[1,2] Bacterial functional amyloids are invariantly extracellular, acting as scaffolding devices in building biofilms, three-dimensional networks that serve to colonize solid surfaces and confer to bacteria persistence against chemotherapy with antibiotics.[3] Amyloids also serve as inactive storage deposits of the antimicrobial microcins.[4] In eukaryotic cells, functional amyloids are either epigenetic determinants of non-Mendelian transmissible characters, as in the case of yeast prions,[5] or scaffolding modules, as for mammalian Pmel17 in melanocytes;[6] or even regulators of long-term synaptic potentiation, as proteins of the CPEB family from mollusks to mice.[7] Among functional amyloids, the ability of the *Escherichia coli* curli system (CsgABC/CsgDEFG) to export nearly any protein tagged to a CsgA-derived secretion signal has enabled the extracellular screening of the amyloidogenic potential of whole proteomes and also of anti-amyloidogenic compounds.[8,9]

The lack of natural intracellular amyloid proteinopathies in bacteria and the fact that expressing any amyloidosis-related human protein in *E. coli* leads to the formation of inclusion bodies, as many other heterologous mammalian proteins do, initially provoked the dismissal of bacteria as model organisms for studies on protein amyloidoses. Furthermore, although inclusion bodies exhibit amyloid features, they do not hamper bacterial viability in a significant way.[10] Interestingly, aggregation is a natural resource in some bacterial proteins in which irreversible structural changes are used to transit across distinct, mutually exclusive, functional states.[1] This is the case of the replication protein RepA of the *Pseudomonas* plasmid pPS10,[11] which undergoes a transition through three association states, each of them linked to a defined function: from stable soluble dimers (transcriptional repressors of *repA* gene expression), RepA dissociates into metastable monomers (acting as plasmid DNA replication initiators), which then aggregate as oligomers that inhibit new rounds of DNA replication (by holding together two plasmid molecules through their replication origins).[12-15] In all these cases, binding to distinct DNA sequences in the plasmid triggers allosteric conformational changes that affect the structure of the N-terminal dimerization winged-helix domain (WH1).[16] While tracking the molecular basis for the functional aggregation of RepA, in 2007 we reported that its isolated WH1 domain, in its mutant variant A31V, was able to assemble into amyloid fibers, built on the core amyloidogenic stretch L_{26}VLCAV-SLI$_{34}$ and upon a conformational change promoted by transient WH1 binding to a short plasmid-specific dsDNA sequence.[17] We proved that, similarly to its action enabling DNA replication initiation through RepA monomerization,[13,16] dsDNA acted as an allosteric effector on RepA-WH1 amyloidogenesis by enhancing its assembly into amyloid fibers.[17] Furthermore, we showed that a small organic molecule (S4-indigo) was able to inhibit such a process by competing with DNA for the binding to RepA-WH1 *in vitro*.[18] Aiming to characterize RepA-WH1 amyloidogenesis *in vivo*, we discovered that expressing fusions of the bacterial protein to a fluorescent protein tracer (mCherry/mRFP), thus replacing the natural C-terminal WH2 domain in RepA,[12,13] generated in *E. coli* a synthetic amyloid proteinopathy that severely reduced

bacterial proliferation and finally led to cell death.[19] These *in vivo* generated aggregates templated the amyloid conformation on soluble RepA-WH1 molecules *in vitro*,[19] as they also did in a mutant variant-specific way *in vivo*,[20] a signature for prion and prion-like proteins. These findings were already discussed in another publication in this journal dated in 2011,[21] and thus will not be treated extensively again here. Instead, we will now review novel results recently reported on the RepA-WH1 prionoid and provide additional insight on the topic.

Structural Polymorphism in RepA-WH1: from Amyloid Nano-Springs and Tubes to Chimeric Yeast Prions

Structural polymorphism, i.e. the alternative ways in which a particular macromolecule can arrange its tertiary and/or quaternary structures, is an intrinsic feature of amyloid assemblies.[22] Polymorphism has been described from the subtleties of the packing of protein side chains and strands in cross-β assemblies to the pitch of twisted amyloid fibers.[23,24] Moreover, the ways and strengths in which phenotypes associated to a particular amyloidogenic protein show up, either in disease or within a physiological function, bear an intimate relation with polymorphism.[25,26]

Recent electron microscopy (EM) and atomic force microscopy (AFM) studies on the amyloid fibers, assembled *in vitro* by templating on soluble RepA-WH1 molecules the amyloid conformation present in the RepA-WH1-mCherry aggregates purified from bacteria, revealed that the characteristic $\approx$25 nm-wide amyloid fibers are actually composed of several coiled filaments, each of them with $\approx$4 nm width (Fig. 1A).[27] In turn, these filaments consist either in a single or a double thread of RepA-WH1 molecules that, with the limitations imposed by the low resolution of the EM reconstruction, are built by distorted monomers. This was inferred from the loose fit of a model based on the crystal structure of a replication-competent monomeric WH1 domain into the EM volume, as well as from circular dichroism (CD) spectra showing an increase in β-sheet structure upon RepA-WH1 assembly, as expected in any amyloidogenesis.[27] Polymorphism is thus

FIGURE 1 (*See next page*). Overview of RepA-WH1 amyloidogenesis, remarking hierarchical assembly *in vitro* (A) and phase transitions *in vivo* (B,C). (A) Stable dimers of RepA-WH1 (dWH1) undergo a structural transformation upon transient, low affinity binding to dsDNA, thus resulting in metastable, aggregation-prone monomers (mWH1*).[13] The core of the WH domain is colored cyan, whereas segments showing significant conformational changes are in blue. The amyloidogenic peptide L_{26}VLCAVSLI$_{34}$ is depicted in red, with the side-chain of the hyper-amyloidogenic mutant residue A31V shown as spheres.[17] Binding of dsDNA (yellow) to dWH1 disrupts the dimerization interface, thus generating partially unfolded mWH1* monomers which assemble as helical amyloid tubular filaments.[17,27] Binding of RepA-WH1 to dsDNA, and thus amyloidogenesis, can be competed by molecules of S4-indigo (spheres),[18] whereas the conformation specific antibody B3h7 (magenta) inhibits the assembly of RepA-WH1 oligomers into filaments.[38] Filaments further associate laterally and coil to generate the mature amyloid fibers (background EM).[17,27] (B) Electron micrograph showing an ultra-thin section through an *E. coli* cell incubated with the B3h7 antibody (arrows/dots: gold-conjugated secondary anti-mouse antibody), which reveals the preferential location of pre-amyloidogenic RepA-WH1 aggregates at the nucleoid (N; green dashed line).[38] A fluidized C-type aggregate hydrogel is also outlined (cyan dotted line).[39] (C) *E. coli* cells growing in microfluidic channels and expressing the prionoid.[39] RepA-WH1 amyloid aggregates show two distinct appearances, i.e. single comet-like (C) aggregates behaving as a fluidized hydrogel that readily splits on cell division; or multiple globular (G) cytotoxic aggregates with the compactness characteristic of typical amyloid plaques.[39] While the phase transition from the C to the G aggregates occurs spontaneously *in vivo*, the reverse uphill transition is promoted by a single cell factor: the Hsp70 chaperone DnaK.[39]
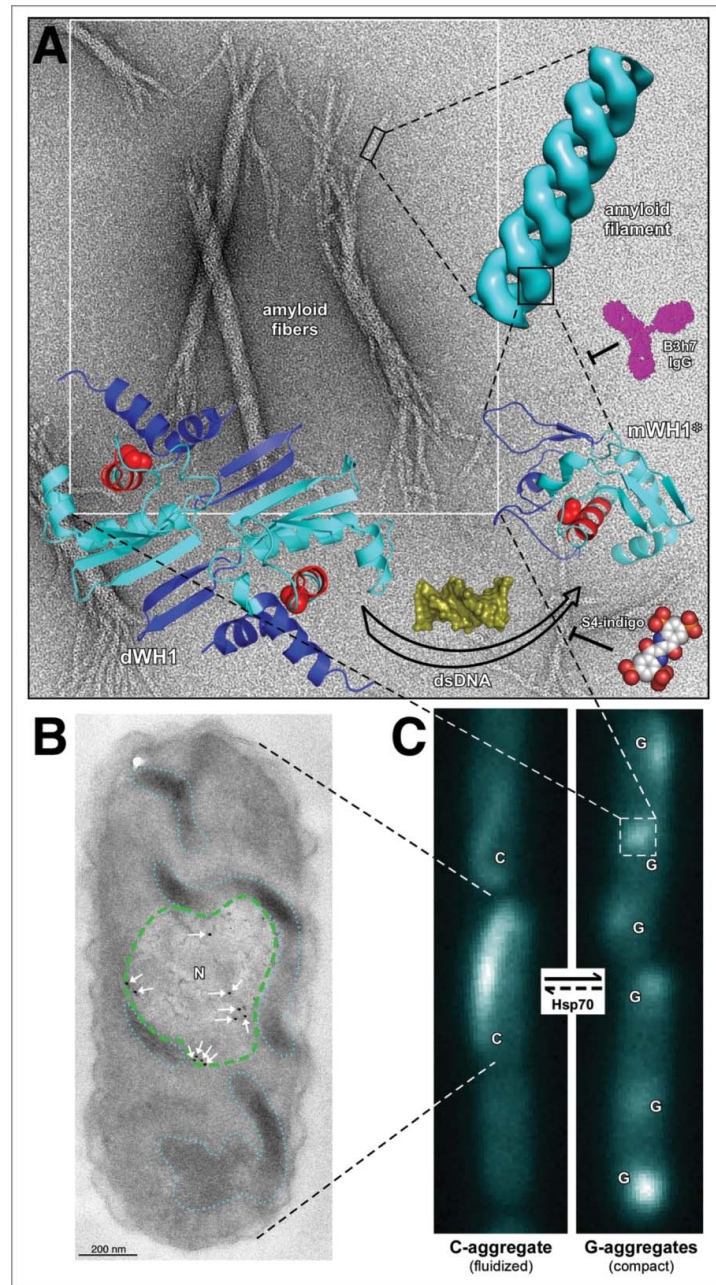
manifested at three levels of increasing complexity: the number of threads that constitute the amyloid filaments (either one or two); the number of filaments per fiber bundle (mode value, 6); and variations in the pitch (on average, 64 nm) of the superhelix that results from twisting several filaments into the mature fibers. Disregarding if the assembly of the fibers was triggered by dsDNA or by templating with purified RepA-WH1-mCherry seeds, and by either leaving the samples to stand in the fridge or agitating them at higher temperatures, which respectively retards or accelerates amyloidogenesis, the fibers generated exhibit

the aforementioned polymorphism.[27] Interestingly, the overall architecture of the twisted RepA-WH1 amyloid filaments can be described as hollow springs (single) or tubules (double filaments) with an axial cavity with a diameter around 2.5 nm. The latter is close to the average dimension of a similar cavity in the short tubular proteotoxic oligomers assembled by α-synuclein.[28] If this is a mere coincidence or if it points to a common mechanism of cytotoxicity for both proteins (e.g., the assembly of pores at membranes) remains to be determined.

Regarding the lateral association of RepA-WH1 filaments, it is known for other amyloid fibers that divalent anions, in particular SO_4^{2-}, play a role in bridging them together, side-by-side.[28-30] In the crystal structure of a RepA-WH1 dimer (PDB entry 1HKQ),[13] two symmetry-related molecules were in contact through a PO_4^{3-} ion, which established a triple salt bridge between Lys85 in one molecule and Lys62 and His63 in the other. During the crystallization attempts, it became evident that incubation with monovalent anions (such as Cl^- or acetate) did not result in crystal growth, whereas SO_4^{2-} rendered poorer diffracting crystals, although this anion yielded the best fibers. The dependence on multivalent anions for RepA-WH1 crystallization matches their requirement in the lateral association of amyloid filaments to build the mature fibers. This could be interpreted as resulting from the overall electropositive charge of the RepA-WH1 molecule,[31] because lowering the ionic strength enhances the dissociation of the fiber ends to the point of releasing their constituent filaments.[27]

What we have learnt on the structure of RepA-WH1 fibers opens an intriguing possibility: that the kind of assembly that monomers of this protein build upon amyloidogenesis could bear some relation to the regulatory complexes of the full-length RepA protein that inhibit new rounds of amplification in recently replicated plasmids.[15] This hypothesis, that needs experimental support, would imply that such regulatory complexes would be a new case of functional amyloid and so the cytotoxic RepA-WH1 prionoid would be the outcome of decoupling amyloidogenesis from its natural linkage to WH2 and plasmid replication,[12,13] thus unveiling the proteotoxic potential typical of amyloids.

Aiming to test *in vivo* the amyloidogenic potential of the $L_{26}VLCAVSLI_{34}$ stretch in RepA, 1-5 repeats of this sequence were used to replace, following the design of a parallel superpleated β-structure,[32] the oligopeptide Q/N-rich repeats at the N-terminal domain yeast protein Sup35p that, in its aggregated state, constitutes the [*PSI*$^+$] prion.[33] As previously proved for a number of other mutant and heterologous sequences,[34-36] the resulting chimeric protein enables an epigenetically inheritable prion, named [*REP-PSI*$^+$],[33] capable of reading-through the stop codon UAG, the phenotype characteristic of [*PSI*$^+$],[5] provided that at least three repeats of the bacterial-related sequence were present.[33] The simultaneous presence of hydrophobic (RepA-WH1 derived) and polar (from the Sup35p scaffold) sequences in [*REP-PSI*$^+$] results in a gradation of weak prion phenotypes/variants, as assessed by the pink color of colonies and the generation of very large aggregates, as evident in semi-denaturing detergent agarose gel electrophoresis (SDD-AGE).[33] Interestingly, biophysical analyses of these functional prion variants revealed that the presence of the hydrophobic RepA-WH1 repeats within the Sup35p prion domain increased the β-sheet structure while resulting in the formation of oligomers, not fibers as for the wild-type prion. The generation of [*REP-PSI*$^+$] variants, which are mitotically stable in terms of propagation, illustrates the feasibility of engineering phenotypic traits in prions by fully exploiting the structural polymorphism of amyloids, from oligomers to fibers, as a constructive resource.[37]

DNA and Hsp70-Modulated Phase Transitions in RepA-WH1 Amyloidogenesis

The effect of dsDNA as an allosteric effector of protein amyloidogenesis *in vitro* was a seminal discovery in RepA-WH1 research.[17] However, the evidence for a similar process *in vivo* was indirect, i.e., a role of DNA in promoting RepA-WH1 amyloidogenesis in *E. coli* cells was inferred from an increase in the number and size of protein aggregates when the vector

expressing the prionoid carried tandem repeats of the DNA sequence that efficiently promoted amyloidogenesis *in vitro*.[19] The development of a monoclonal antibody (termed B3h7), characterized *in vitro* as specific for a pre-amyloid oligomeric conformation on-pathway to mature RepA-WH1 fibers (Fig. 1A),[38] has recently made possible to unveil, through immuno-electron microscopy (iEM) (Fig. 1B), that in *E. coli* cells expressing the prionoid the very first amyloid oligomeric precursors are found at the bacterial nucleoid,[38] as expected from the known ability of DNA to promote RepA-WH1 amyloidogenesis *in vitro*[17,18] and possibly from the original function of the WH1 domain when, in the whole RepA, controls plasmid DNA replication.[15] It was found, also by iEM, that amyloid particles exceeding ≈ 100 nm in diameter are placed out of the nucleoid territory,[39] as expected if entropic exclusion by the nucleoid were playing a mayor role in the localization of the aggregates. These findings could have implications for the preferential or alternative tropism of amyloid aggregates toward the cytoplasm or the nucleus in some human neurodegenerative proteinopathies, or in the case of proteins such as FUS, whose ability to assemble functional ribonucleoparticles in either location becomes impaired in amyotrophic lateral sclerosis and frontotemporal dementia.[40-42]

Concerning the intracellular inclusions formed by amyloidogenic proteins either in the cytoplasm or in the nucleus of eukaryotic cells, it is remarkable the recent description of the existence of phase transitions between soluble, hydrogel and fibrilar states, in some cases through intermediates with the appearance of liquid droplets.[40-44] Such partitions imply changes in the association state beyond the quaternary structure, generating the so-called quinary structural level, having consequences for the functionality and bioavailability of the macromolecules involved. In many instances, these are proteins bearing domains with amino-acid compositions biased toward low complexities. In the case of Q/N/G-rich domains, phase transitions have been related with the package of mRNA in stress/P-granules and are relevant in amyloidogenesis.[40-42] Interestingly, albeit RepA-WH1 lacks such simplified amino-acid composition and has a very stable fold in its native

state, when assembled as cytoplasmic aggregates *in vivo*, exhibits similar phase transitions, the first instance in which this has been observed in bacteria.[39] In these experiments, a combination of fluorescence microscopy and microfluidics was used, because this enabled monitoring bacterial proliferation and the dynamics of RepA-WH1 aggregation at the level of multiple single cell lineages, for many generations (up to 200) and under controlled environmental conditions. For studies on amyloidosis, surveying the physiology of bacteria in microfluidic devices surpasses the approaches based on agarose pads in terms of the number of generations achievable before the cessation of growth, the feasibility to track lineages and the ease to inject into, or extract from, the cultures diverse molecules of interest. Two clearly distinct RepA-WH1 aggregates became evident at the microfluidic device (Fig. 1C): i) multiple globular, compact cytotoxic (G) particles that inhibit cell division; ii) a single, comet-shaped (C) aggregate that appears to be fluidized, i.e. elongates, contracts and undulates along the cell axis and readily splits between the daughter cells on cytokinesis.[39] The outcome is that while bacteria expressing the G-type aggregates show a tendency toward filamentation and cells die after a few generations, those bearing the C-type aggregates keep dividing indefinitely at a reasonable rate. In an isogenic population expressing RepA-WH1, bacteria at distinct microfluidic channels propagate for generations one or the other type of aggregates, although interconversion (more commonly C to G) occurs at low rates.[39] We also explored the effect of the Hsp70 chaperone DnaK on RepA-WH1 amyloidogenesis, because it is central in counteracting protein aggregation in bacteria and a role for DnaK in the activation of RepA-type proteins for plasmid replication was recognized for long.[11] It is the activity of DnaK, i.e., its expression induced from a regulated promoter *vs.* its specific inhibition with myricetin, what determines the balance and switching (phase transition) between both kinds of aggregates: DnaK promotes the conversion of most cells to the elongated phenotype, whereas its inactivation results in the population dominated by the globular phenotype.[39] iEM studies revealed that DnaK is preferentially located at the aggregate with an elongated and fluidized appearance, which

constitutes a territory with a clearly different density toward the electrons compared with that observed for the globular aggregates.[39] Therefore this Hsp70 chaperone, most likely through its aggregate remodeling activity, is the factor determining quinary structural phase transitions for RepA-WH1 in the bacterial cytoplasm. On the contrary, the bacterial ClpB (Hsp100) chaperone has no apparent contribution to this effect. Coming to the point, the work carried out with our synthetic bacterial model prionoid suggests that the long sought cell factor(s) that would ameliorate amyloid cytotoxicity in human proteinopathies might be Hsp70 chaperones by promoting the assembly of pro-amyloidogenic proteins as non-fibrilar hydrogels.

FURTHER PERSPECTIVES

Ongoing research on the bacterial prionoid RepA-WH1 aims to trace a scheme of the molecular basis for amyloid cytotoxicity in such a minimal host as *Escherichia coli*, with the ambition of providing clues on the undeniably more complex mammalian amyloid proteinopathies. Such endeavor spans from a systems biology perspective, to highlight gene networks and metabolic pathways related to amyloid disease, to bottom-up approaches based on reconstructive, cytomimetic biochemistry performed with lipid vesicles.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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