Hexapeptide Tandem Repeats Dictate the Formation of Silkmoth Chorion, a Natural Protective Amyloid

Paraskevi L. Tsiolaki[†], **Nikolaos N. Louros**[†] **and Vassiliki A. Iconomidou**

Section of Cell Biology and Biophysics, *Department of Biology, School of Sciences, National and Kapodistrian University of Athens, Panepistimiopolis, Athens 157 01, Greece*

Correspondence to Vassiliki A. Iconomidou: *veconom@biol.uoa.gr*
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Abstract

Silkmoth chorion is a fibrous structure composed mainly of two major protein classes, families A and B. Both families of silkmoth chorion proteins present a highly conserved, in sequence and in length, central domain, consisting of Gly-rich tandem hexapeptide repetitive segments, flanked by two more variable N-terminal and C-terminal arms. Primary studies identified silkmoth chorion as a functional protective amyloid by unveiling the amyloidogenic properties of the central domain of both protein families. In this work, we attempt to detect the principal source of amyloidogenicity of the central domain by focusing on the role of the tandem hexapeptide sequence repeats. Concurrently, we discuss a possible mechanism for the self-assembly of class A protofilaments, suggesting that the aggregation-prone hexapeptide building blocks may fold into a triangle-shaped β-helical structure.

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Introduction

Protein misfolding is intimately related to a group of human diseases such as Alzheimer's Disease, type II diabetes or spongiform encephalopathies, commonly reported as amyloidoses [1]. As the key hallmark of such pathologies, amyloids are formed by otherwise soluble proteins, which under denaturing conditions undergo conformational re-arrangements and obtain the same well-defined three-dimensional (3D) cross-β conformation [2]. Curiously, evolution maintained the cross-β architecture for numerous biological functions, and thus, amyloids were also mapped to organisms spanning from bacteria to humans, as functional amyloids [3, 4]. Over the last 20 years or so, there have been major advances in the study of functional/protective amyloids as beneficial structures of the physiology of several organisms [1, 3]. At the same time, a growing number of studies indicates that short stretches of the misfolded proteins (called "amyloidogenic determinants" or "hot spots of aggregation") may nucleate protein aggregation and amyloid formation [5–7]. Such stretches, possessing noteworthy self-aggregation properties, either mediate amyloidogenesis in several pathologies [8–11] or, in certain cases, are important structural elements, "hidden" after millions of years of evolution in protein sequences [12].

The eggshell of many insects and fish eggs has been the subject of numerous investigations at the cellular and molecular level [13]. Chorion is the major component of the eggshell of all insects and fish eggs and supports both the development of the oocyte and the survival of the growing embryo [14]. The layer of chorion in silkmoths consists of more than 200 different structural proteins, which are secreted to cover the surface of the oocyte and account for more than 95% of its dry mass [15, 16]. Silkmoth chorion proteins (SCPs) are categorized into two major comparable protein classes, namely, A and B families [17]. According to extensive structural studies, both families exhibit a tripartite structural model: an evolutionary conserved central domain and two flanking terminal domains, namely, "N-arm" and "C-arm" [18, 19] (Fig. 1a). The central domain (Fig. 1a, cA peptide) is conserved in both classes and contains characteristic hexapeptide tandem repeats (Fig. 1a, stars). Conversely, N- and C-domains (Fig. 1a, N-arm and C-arm) display high

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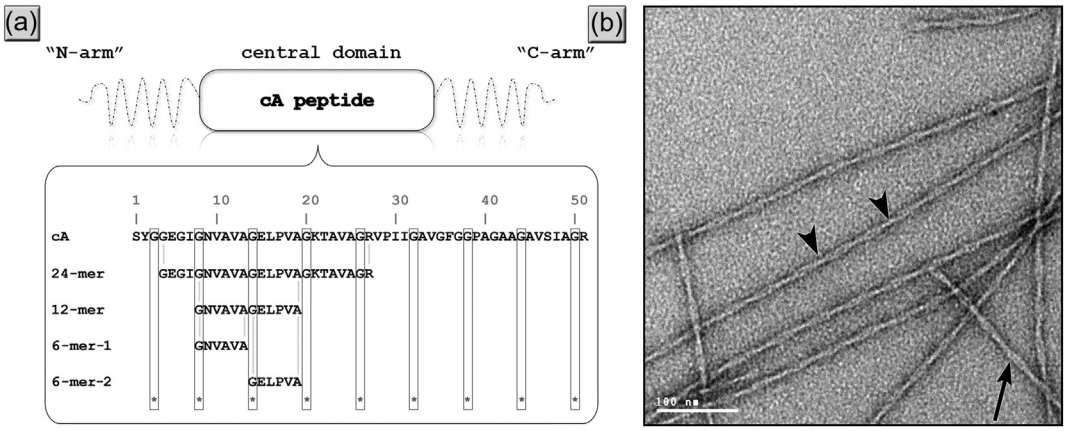

Fig. 1. The central domain of SCP of the A family self-assembles into amyloid-like fibrils. (a) The A family of SCPs consist of three domains: the central domain (cA peptide), the N-terminal flanking domain (N-arm) and the C-terminal flanking domain (C-arm). The primary sequence of the conserved central domain of SCPs of the A family is also presented. 24-mer, 12-mer, 6-mer-1 and 6-mer-2 are synthetic peptide sequences that were synthesized and studied by our group in previous studies [20] and in the present work. Tandemly repeating Gly residues, comprising the central domain, are marked with a star. (b) Electron micrograph of negatively stained amyloid-like fibrils, derived by self-assembly from an aqueous solution of the cA peptide. Fibrils are 90 Å in diameter and have a double-helical structure. The pitch of the helix (black arrowheads) is approximately 920 Å. The black arrow points a pair of protofilaments each ~40 Å in diameter.

variability and consist of repetitive short peptides that do not appear in the central domain [19]. Chorion complexity and ultrastucture were used as a model system in several fields of biological research. Extensive structural and sequencing studies of the component proteins tried to elucidate all mechanisms that regulate the production of chorion proteins [13, 15]. However, technical difficulties in producing individual chorion proteins in large enough amounts and of sufficient purity resulted in slow experimental progress in this area. A thorough understanding of the hierarchical assembly of SCP into fully formed fibrils has been achieved by computational analyses of the chorion amino acid periodicities and molecular modeling [12, 13, 19]. Here, we attempt to give a brief overview about the amyloidogenic nature of SCPs by assessing all previously conducted studies and by further updating experimental and computational data in the field.

Chorion is a natural protective amyloid

Early electron microscopy studies demonstrated that the ultrastructure of silkmoth chorion contains arrays of packed fibrils, forming a biological analogue of a cholesteric liquid crystal with an unknown, at the time, molecular architecture [13, 14, 21]. Valuable insights into the nature of SCP were first introduced from structural studies on the central conserved domain (herein called cA peptide) [12]. Utilizing a consensus sequence representative of about 30% of all the proteinaceous material in the eggshell, Iconomidou *et al.* reported, back in 2000, the existence of a natural protective amyloid (Fig. 1b). This finding was then supported by numerous studies on silkmoth chorion, such as X-ray fiber diffraction [14], Fourier-transform infrared spectroscopy (FT-IR) and laser Raman spectroscopy [18, 22], all of which indicated a β-sheet conformation as the dominant secondary structure of its constituent proteins. The identification of a fibril forming 51-residue-long peptide (cA peptide) as a crucial functional domain in SCPs provided an entirely different perspective for amyloidogenicity, by associating, for the first time, the amyloid conformation with the native conformation for chorion protein fibrils. Evidence at experimental level revealed that the central domain of the A family of SCP (Fig. 1a, cA peptide) self-assembles forming amyloid-like fibrils *in vitro* (Fig. 1b), under a great variety of conditions [12]. Later studies reported the self-assembly properties of a 24-residue long peptide, half of the central domain of the A family [20] (Fig. 1, 24-mer), suggesting that amyloid fibril formation in SCP is possibly sustained by fractions of the conserved central domain. The idea of silkmoth chorion as a natural protective amyloid was further supported by relevant experiments on representative peptide-analogues, derived from the central domain of the B family [23, 24].

Identifying the shortest amyloid-forming peptide in SCPs

Taking a step back to assess the currently available chorion protein sequences of the A family, it is clear that the amyloidogenic cA peptide sequence overall represents the highly conserved central domain region of this broad protein family (Fig. 2).

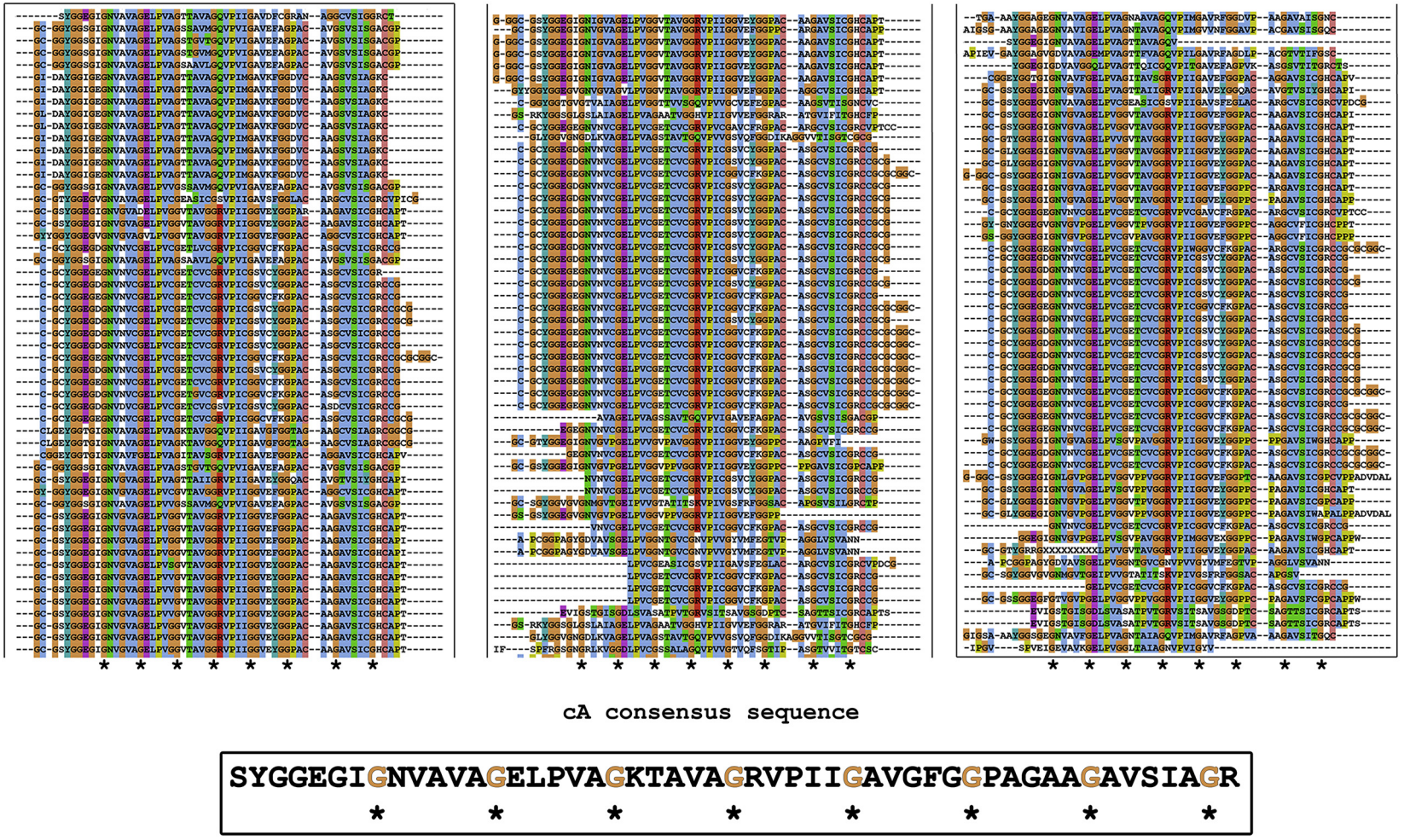

Fig. 2. Multiple sequence alignment of the central domain of 156 proteins of the silkmoth chorion A family. Each line represents an entry, obtained from LepChorionDB [25]. The presence of conserved tandem hexapeptide repeating motifs enriched in Gly residues is marked with stars. The consensus cA peptide sequence is also provided. All central domains were aligned with ClustalW [26] and visualized with Jalview [27]. The multiple sequence alignment is based on the default ClustalX [28] color scheme (blue, hydrophobic residues; red, positively charged residues; magenta, negatively charged residues; green, polar residues; cyan, aromatic residues; pink, cysteines; orange, glycines; yellow, prolines). Gaps or residues that do not meet the default criteria of ClustalX are shown in white.

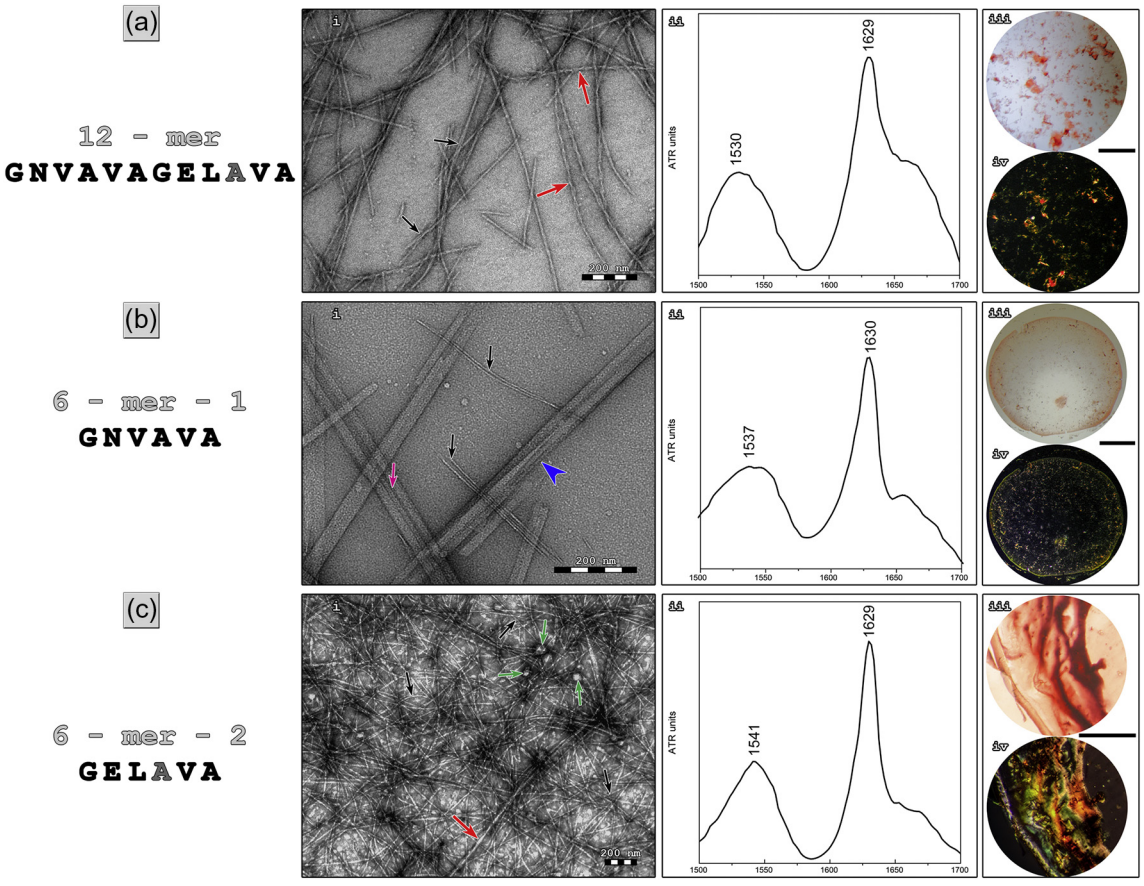

Fig. 3. The amyloidogenic properties of the 12-mer, 6-mer-1 and 6-mer-2 peptides. Electron micrographs of mature amyloid-like fibrils from the 12-mer (a-i), 6-mer-1 (b-i) and 6-mer-2 (c-i) peptides, respectively. The samples were negatively stained with 2% uranyl acetate. Arrows show distinct polymorphic, fibrillar structures (see main text). The bar represents 200 nm. ATR FT-IR (1500–1800 cm^{-1}) spectra, obtained from suspensions of fibrils, produced from the 12-mer (a-ii), 6-mer-1 (b-ii) and 6-mer-2 (c-ii) peptides, cast on a flat stainless-steel plate and left to air-dry slowly, at ambient conditions. Photomicrographs of the 12-mer (a), 6-mer-1 (b) and 6-mer-2 (c) peptide fibrils stained with Congo red: bright field illumination (iii), crossed polars (iv). The yellow-green birefringence, characteristic for amyloid fibrils, is clearly seen under crossed polars in all cases (a-iv, b-iv and c-iv). The bar represents 400 μm. Formation of amyloid-like fibrils, negative staining, Congo red staining and FT-IR spectra manipulations were performed for all three peptides as described by Iconomidou *et al.* [12, 20].

The presence of conserved Gly-rich tandem hexapeptide repeating motifs was validated through an updated alignment of more than 150 currently available class A protein sequences, obtained from LepChorionDB (Fig. 2, stars) [25]. As aforementioned, both the entire cA domain and the peptide fragment, comprising four hexapeptide repetitive segments (Fig. 1a, 24-mer), are capable of forming amyloid-like fibrils, suggesting that the amyloid-forming properties are hidden in shorter fragments of the central domain.

Aiming to track the shortest amyloidogenic fraction in SCP, we experimentally tested peptide-analogues derived from sequence fragments of the cA peptide (Fig. 1a). First, a 12-residue-long peptide (Fig. 1a, 12-mer) was rationally designed, representing a core conserved region of both the half of the 24-mer amyloidogenic peptide [20] (Fig. 1a, 24-mer) and the quarter fragment of the central cA domain. This 12-mer peptide (GNVAVAGELPVA to GNVAVAGELAVA), was chemically synthesized, having a point substitution of Pro to Ala, in order not to disrupt the structure that is going to be formed. An aliquot of the lyophilized 12-mer peptide was incubated in a concentrated aqueous solution (pH 5.75) at room temperature for 7 days. Transmission electron microscopy confirmed the presence of amyloid-like fibrils, which are ~80–90 Å in diameter (Fig. 3a-i, black arrows). Evidently, single fibrils coil along each other, resulting in well-shaped super-helices (Fig. 3a-i, red arrows), with an axial periodicity resembling cA amyloid-like fibrils (Fig. 1b) [12]. ATR FT-IR spectral acquisitions suggest the presence of β-sheet conformation as the predominant structure of amyloid-like

fibrils of the 12-mer peptide (Fig. 3a-ii). Fibrils derived from the 12-mer peptide were judged to be amyloid-like, since they specifically bind the Congo red dye (Fig. 3a-iii) showing a typical apple-green birefringence, when viewed under crossed polars of a polarizing microscope (Fig. 3a-iv).

After inspecting the amyloidogenic nature of the 12-mer peptide, we took the analysis a step further focusing on the shortest stretches of the consensus cA sequence, namely, the tandem hexapeptide repeats. Accumulating evidence highlights the significance of hexapeptides as more important than that of tetrapeptides and pentapeptides in the formation of the cross-β structure, indicating that when it comes to amyloid aggregation propensity, 6-residue-long stretches are sufficient to nucleate amyloid formation [29, 30]. The two tandem hexamer repeats comprising the 12-mer peptide, namely, GNVAVA and GELAVA (Fig. 1a) present similar aggregation propensity to all the 12-mer, the 24-mer, and the cA peptides, in comparable conditions. The 6-mer-1 peptide forms lamellar nanocrystalls (Fig. 3b-i, blue arrowhead), containing thin single protofibrils (~50–60 Å), with a tendency to be associated laterally. A magenta arrow points the thinnest single fibril, approximately 50 Å in diameter (Fig. 3b-i, magenta arrow). Similar crystalline structures have been observed and subsequently used to determine the assembled structure of short amyloidogenic segments by X-ray crystallography [31]. The 6-mer-2 stretch self-aggregates into fibrils with typical amyloid morphological features and creates networks of densely arranged fibrils (Fig. 3c-i). The majority of the 6-mer-2 fibrils are estimated to have diameters of about ~60–70 Å (Fig. 3c-I, black arrows), whereas in some cases, fibrils showed a similar tendency to create double helical structures, as also reported for the 12-mer (Fig. 3c-i, red arrow), the 24-mer [20] and the cA peptide (Fig. 1b) [12]. Interestingly, the co-existence of spherulites (Fig. 3c-i, green arrows), which have been previously reported as the initial step of the cA fibrillogenesis [32], reveals a crucial common feature between the entire cA region and the short hexapeptide repeat. Subsequently, ATR FTIR spectra of thin films cast from suspensions of the amyloid-like fibrils of both hexamers (Fig. 3b-ii and c-ii) show prominent bands at wavelengths indicative of the β-sheet conformation, validating the amyloid-like architecture of the formed aggregates. Ultimately, Congo red staining combined with polarizing microscopy revealed yellow to apple-green birefringence for both the 6-mer-1 (Fig. 3b-iv) and 6-mer-2 (Fig. 3c-iv), when placed under crossed polars.

To sum up, it appears that the key amyloidogenic properties of the conserved cA domain of SCP are possibly encrypted in the tandem hexapeptide repetitive segments of the central conservative region. All experimentally tested peptides form similar amyloid fibrils with comparable morphologies, a process that may be mediated by the same liquid-crystalline intermediates (spherulites) detected for the first time for the cA peptide [32]. This observation implies that the distinct amyloidogenic features of SCP stem from the "hexapeptide repeat fingerprints" of the central domain following the example of several other amyloid-forming proteins, containing tandem sequence repeats [9, 33].

Aggregation-prone hexapeptide repeats may direct SCPs toward a β-solenoid 3D fold

The presence of tandem repeats, composing the amyloid-forming region of SCP, is a striking feature that is commonly shared by proteins associated with the formation of functional amyloids [33–35]. Several studies have previously suggested that tandem repeats comprising 5–20 residues usually possess a defining structural role and are commonly associated with the formation of closed super-helical β-solenoid structures [34, 35]. β-Solenoids are fibril-like extended structures of stacked β-strand components that are usually formed by a single or multiple repeats and, thus, are compatible to the overall β-structure of amyloids [36, 37]. These structures incorporate tight turns and are frequently stabilized by organized hydrogen-bond networks. β-Solenoids are regularly found to sequences associated with the formation of functional amyloids,

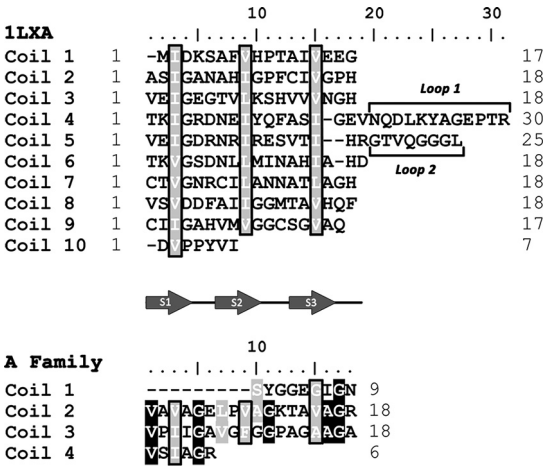

Fig. 4. cA domain of the SCP share similar periodicities to UDP-*N*-acetylglucosamine acyltransferase from *E. coli*. The UDP-*N*-acetylglucosamine acyltransferase from *E. coli* (PDB ID: 1LXA) [44] was used as a template to build a 3D β-solenoid model for the cA domain of the SCP. Evidently, both UDP-*N*-acetylglucosamine acyltransferase and cA sequences share periodicities of the same size (18 residues) and have sequence patterns with similar physicochemical properties.

which are more enriched in polar (Q/N rich) as well as Gly and Pro residues, such as in the case of SCP [34, 35].

Several functional amyloids have been associated with a β-solenoid-like fold, starting with the 3D determined structures of antifreeze proteins [38]. A similar fold was determined for HET-s that forms functional amyloids in *Podospora anserina* [39], whereas similar structural models have been proposed for Pmel17 [36] and prions [37]. Finally, β-helical folds have also been proposed for curli proteins of several bacterial strains, such as *Escherichia coli* [33], *Salmonella enteritidis* [40] and *Pseudomonas* [41], which are responsible for the formation of biofilms. SCP repeats (Fig. 1a, stars) share several characteristics with the previous amyloid-forming sequences, since they are short periodicities enriched in Gly and Pro residues, with relatively low hydrophobic content. Evidence of the β-solenoid propensity of SCP is provided by analysis with REPETITA, an algorithm that specializes in identifying β-solenoid forming sequences [42]. Analysis of the central domain of class A chorion proteins suggests a β-solenoid structure with high certainty, judging by the distance of the predictions from the optimal line separating solenoid-forming from non-solenoid sequences (default REPETITA output, data not shown).

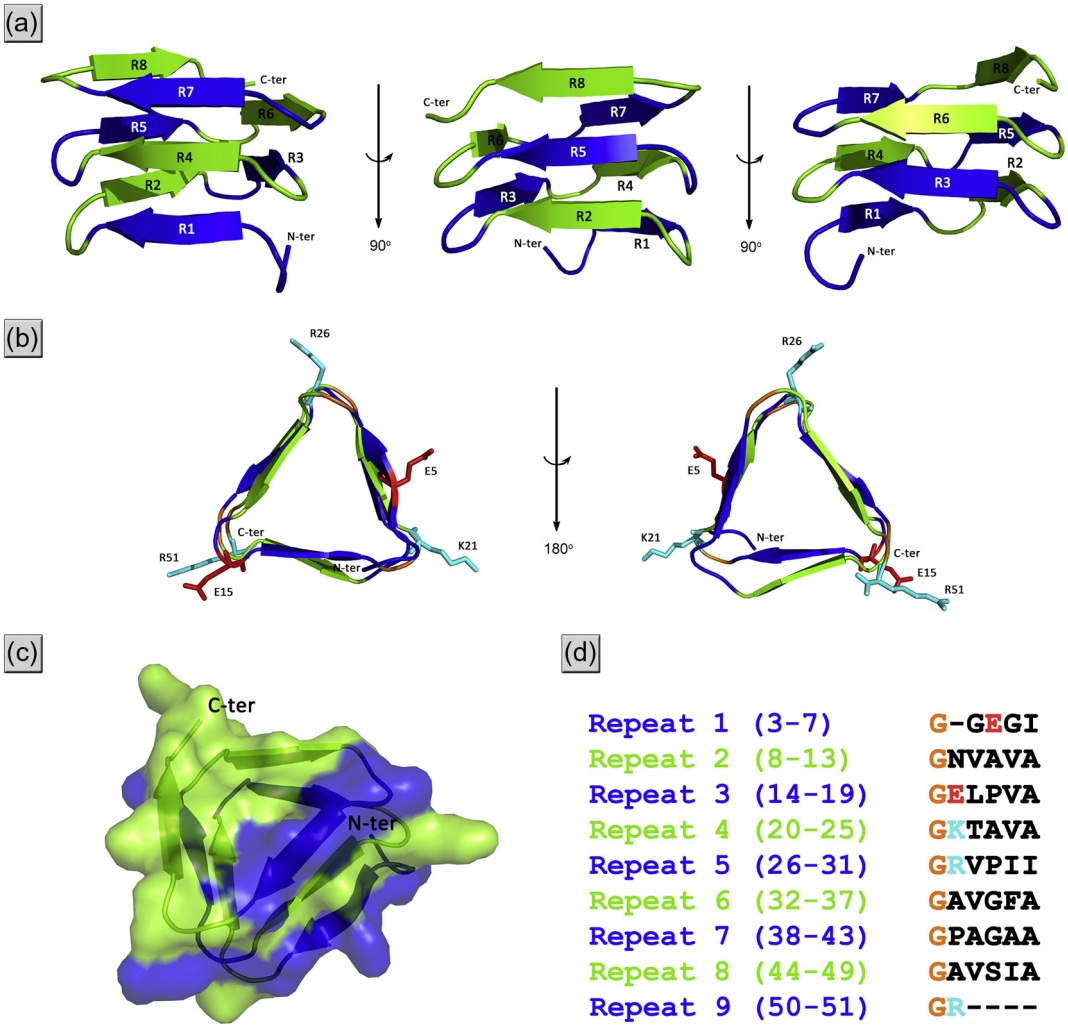

Fig. 5. Structural insights into the central domain of A family silkmoth proteins. (a) Three-dimensional model of the left-handed β-helical structure of the consensus cA domain (rotated 0°, 90°, 180°). Successive repeats (R1–R8) are colored green and blue, respectively. (b) Invariant Gly and charged residues are located at the connecting β-turns. Charged residues are shown as sticks. (c) Surface representation of the triangle-shaped β-helix. (d) The hexamer tandem repeats of the consensus cA central domain sequence. Gly residues are colored orange, whereas charged residues are colored red and cyan (N-ter: N-terminal region, C-ter: C-terminal region). The structure was constructed utilizing modeler 9v2 [45] and was subsequently minimized using the MMTK toolkit [46].

To investigate a possible β-solenoid fold for the central domain of class A SCPs, we scanned through RepeatsDB [43]. Different template structures from RepeatsDB were utilized, so as to computationally investigate the most compatible 3D conformation that fits to our derived experimental results. As a result, a 3D β-solenoid fold was modeled by threading the sequence of cA over the β-helical structure of UDP-*N*-acetylglucosamine acyltransferase from *E. coli* (PDB ID: 1LXA) [44] (Fig. 4). Our selection was based on the fact that both sequences share periodicities of the same size, a defining factor of the β-solenoid fold, in addition to a similar hydrophobicity pattern (Fig. 4). The derived structure was constructed utilizing modeler 9v2 [45], minimized using a steepest decent methodology included in the MMTK toolkit [46] and optimized following the example of recent β-helical computational design methodologies [47–49]. The final structure was evaluated using DSSP and the WHAT-IF portal [50] (Fig. 5).

The cA model structure adopts a left-handed parallel β-helix conformation (Fig. 5a) with triangle-shaped (T-shaped) cross sections composed of three hexapeptide repeats (Fig. 5b) that stack along the individual sides of the solenoid axis (Fig. 5c). The highly conserved Gly periodicities (Fig. 2, stars) have a central role in the overall stability of the structure. Glycines occupy positions in the tight turns that connect the successive 4-residue long β-strands, which in turn incorporate invariant β-strand-forming hydrophobic positions that interdigitate toward the inner tight hydrophobic core (Fig. 5d). Taking advantage of the fact that T-shaped solenoids have been shown to share a tendency to organize into trimeric polymers, a similar polymer super-structure was investigated for our modeled structure (Fig. 5a), utilizing template crystal contacts [35]. In this arrangement, conserved polar and charged side-chain positions are stacked toward the exterior and possibly stabilize the trimeric interaction through the formation of salt bonds (Fig. 6a) or through the formation of a hydrogen bond network, a common feature of β-solenoids [35]. A less likely alternative suggests that the exposed charged side chains may contribute to the formation of isopeptide bonds (Fig. 6b, circles), as an evolutionary residual of a previously proposed hardening mechanism in fish chorion [51, 52]. Successive stacking of cA conformers along their β-helical axis can in this trimeric arrangement lead to the formation of a cross-β superstructure with a diameter of 60–70 Å (Fig. 6c). It is likely that aggregation-prone hexapeptide hotspots from both sides of cA peptide could simulate the formation of the protofilaments that were experimentally determined to be formed by the cA peptide (Fig. 1b).

Perspectives

Chorion challenges several aspects of biological research, since it has served as an innovative model system [14, 53]. After millions of years of molecular evolution, chorion has been exploited by natural selection to protect the developing embryo from a wide range of environmental hazards [13, 14]. Structural studies on SCPs of the A class were the first to propose that amyloid proteins may even be

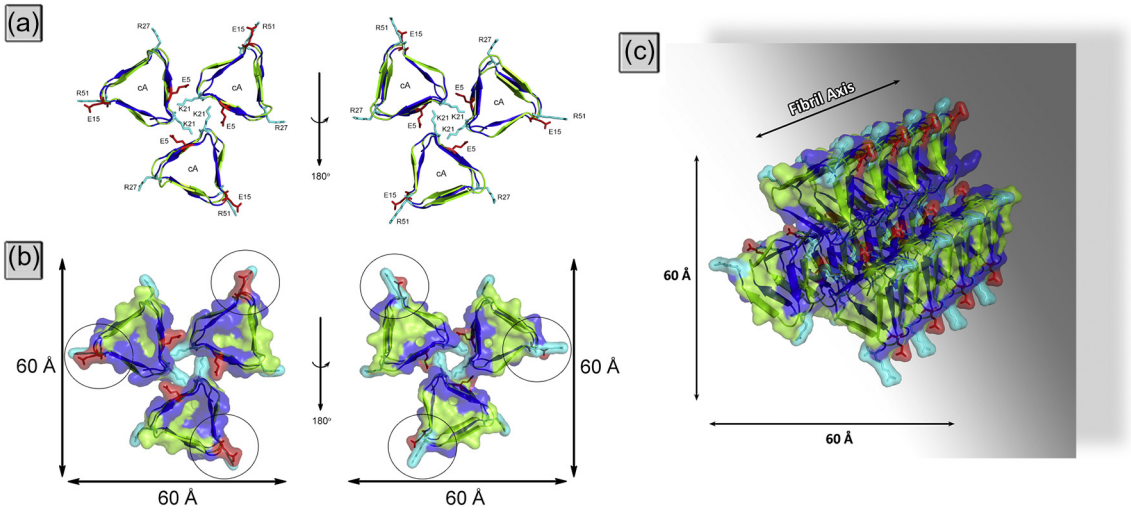

Fig. 6. The trimeric arrangement of the cA protofilament. cA protofilaments, approximately 60 Å in diameter, consist of β-solenoid trimers, stabilized by the presence of intermolecular salt bonds. Cartoon representation (a) and surface representation (b) of the β-solenoid trimer were modeled, utilizing template crystal contacts [35]. Charged residues are shown as sticks and colored red and cyan. (c) The trimeric arrangement of the cA protofilament may be formed *via* the successive stacking of cA conformers along their β-helical axis.

used toward a beneficial end [12], a notion that was later on extended to protective coats of additional organisms [54–57]. In this work, we attempted to gather information referring to the chorion amyloidogenicity and further determine the shortest possible element from the sequences of SCP that folds and self-assembles forming fibrils similar to those appearing *in vivo* in the structure of silkmoth chorion [14]. Seeing that the amyloidogenic properties of the cA central domain are carried on the 24-mer peptide [20], the 12-mer peptide and the hexapeptides (6-mer-1 and 6-mer-2) (Fig. 1a), we may suggest that the amyloidogenic properties of silkmoth chorion are evolutionarily encoded into tandemly repeating hexapeptides, forming the central domain of SCP [12, 20].

To date, peptide self-aggregation studies have been reported as an excellent starting point for the design of functional amyloid materials [58], since self-assembling peptides have a long history in the development of biologically compatible scaffolds [59], carriers of long-acting drugs [60] or nanostructured films [61] and are also currently used as the means for targeted aggregation technologies [62]. Therefore, apart from chorion biological principles, the impact of chorion proteins as a system may be proved inspiring for deciphering the mechanisms of amyloid formation or for the rational design of highly stable, amyloidogenic molecules with diverse applications.

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†Equally contributing authors.

Present address: N.N. Louros, VIB Switch Laboratory, 413 Department of Molecular Cellular and Molecular Medicine, 414 VIB-KU Leuven, B-3000 Leuven, Belgium.

Abbreviations used:
SCPs, silkmoth chorion proteins; FT-IR, Fourier-transform infrared spectroscopy; 3D, three-dimensional.

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