Amyloid-like fibrils from a peptide-analogue of the central domain of silkmoth chorion proteins

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The structure and self-assembly properties of a 51-residue peptide-analogue of the central conservative domain of silkmoth chorion (eggshell) proteins were studied in detail. This peptide (cA peptide) forms amyloid-like fibrils, under a variety of conditions.

The fibrils bind Congo red and thioflavin T. Negative staining and shadowing showed that the fibrils are twisted, forming double helices. The average width of the basic double helical unit is ~90 Å and the helical pitch of the double helix ~920 Å. Individual fibrils (protofilaments) which super-coil to form the double helix have a diameter of ~30-40 Å. FT-IR and FT-Raman spectroscopy indicated an antiparallel β-sheet type of structure. X-ray diffraction patterns from fibres of the cA-peptide, taken with a double-mirror camera, clearly indicate a "rich", oriented cross-β fibre pattern characterized by a meridional reflection at ~4.66 Å and an equatorial reflection at ~10.12 Å.

Modeling studies suggest that a twisted β-sheet of 4-residue β-strands alternating with β-turns is the basic structural motif of the fibrils. The models are similar to the cross-β structure proposed a decade and a half ago for silkmoth chorion proteins to dictate the helicoidal architecture of intact, native chorions. Thus, it appears that silkmoth chorion is the first well documented case, where amyloid plaques formed from self-assembly of chorion proteins, *in vivo* protect the oocyte and the developing embryo from a wide range of environmental hazards.

Silkmoth chorion is the major component of the eggshell covering the oocyte and the developing embryo. It is a proteinaceous protective and functional layer with extraordinary mechanical properties and an interesting model system in several current areas of biological research: cellular differentiation, molecular evolution and fibrous protein folding and self assembly (for reviews see Regier & Kafatos, 1985; Goldsmith & Kafatos, 1984; Hamodrakas, 1992). Understanding the relationship between structure, function and assembly of its component proteins might be fruitful into the design of novel biomaterials (Hamodrakas, 1992).

Biochemically, silkmoth chorion is surprisingly complex: about 200 proteins have been resolved by two-dimensional gel electrophoresis (Regier & Kafatos, 1985). They were classified into a small number of families or classes: the most abundant are the A's and the B's which together account for almost 90% of the total chorion mass. The gene families that encode these proteins are themselves related and constitute a superfamily with two branches, the α-branch and the β-branch (Lecanidou et al., 1986).

Aminoacid sequence comparisons and predictions of protein secondary structure revealed that chorion proteins exhibit a tripartite structure: a central domain, evolutionarily conserved in each protein class (recognizably homologous among the two main A and B classes) and two flanking N- and C- terminal domains or "arms" (Figure 1). These arms are more variable but they are marked by the presence of characteristic tandemly repetitive short peptides that do not appear in the central domain (Hamodrakas et al., 1982; Hamodrakas et al., 1985).
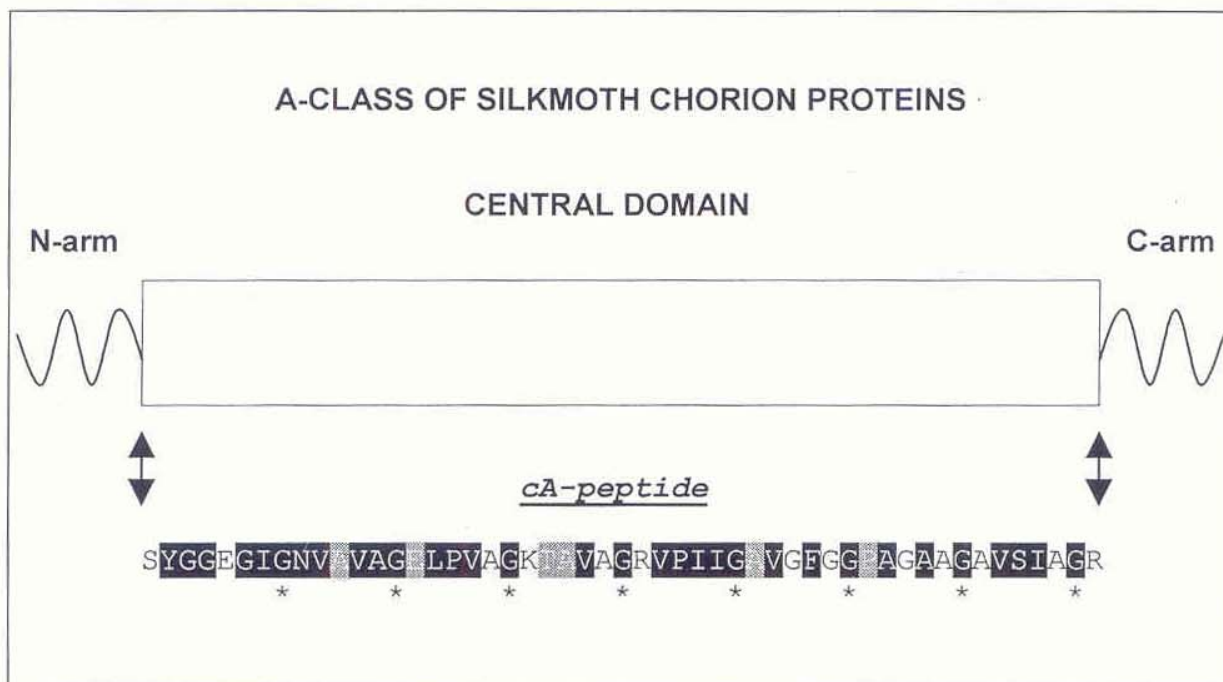

Figure 1. Schematically, the tripartite structure of silkmoth chorion proteins of the A class. A central domain highly conservative and of invariant length, and two more variable flanking "arms" constitute each protein. Characteristic, tandemly repeating peptides are present both in the central domain and in the "arms". (Hamodrakas, 1992 and references therein). The B-class of proteins exhibits a similar tripartite structure.
The aminoacid sequence of the synthetic cA peptide (one letter code), which was designed to be an analogue of the central A-domain is also shown. Invariant glycines repeating every six-residues are marked with an asterisk below the sequence. Black-boxed residues are conservd 100% and gray-boxed residues represent 80% conservative substitutions.

A structural model has been proposed for the central domain and the flanking arms combining data from amino acid sequence comparisons, secondary structure prediction, analysis of amino acid periodicities and modelling (Hamodrakas et al., 1985; Hamodrakas et al., 1988). According to this model chorion proteins adopt a

characteristic antiparallel, most probably twisted, β-pleated sheet structure of four residue β-strands connected by β-turns (Hamodrakas et al., 1985; Hamodrakas et al., 1988), in a cross-β conformation. Evidence from X-ray diffraction, laser-Raman, infrared and CD spectroscopy supports this model (Hamodrakas, 1992 and references therein).

However, because of chorion complexity, it is very difficult to isolate and purify individual chorion proteins suitable for structural studies. Therefore, an alternative approach was chosen in attempts to elucidate principles that govern chorion protein folding and assembly: peptides thought to be representative of certain, structurally important, parts of chorion proteins were synthesized and an effort is currently being made to study their structure both in solution and in the solid state. It is hoped that this analysis will reveal folding and packing modes significant in chorion protein structure and assembly.

One such peptide, a 51-residue peptide, hereafter called cA, is an analogue of the entire central domain of the A class of silkmoth chorion proteins (Figure 1). This peptide is representative for about 30% of all the proteinaceous material in the eggshell. We designed this peptide since the central domains of the class A chorion proteins are highly conserved in both sequence and length and this conservation indicates an important functional role for this structural entity in chorion structure formation. Preliminary laser-Raman and FT-IR experiments (Benaki et al., 1998) showed that the structure of this peptide is predominantly anti-parallel β-pleated sheet both in solution and in the solid state.

Results

The cA peptide was synthesized as described by Benaki et al. 1998. It forms, uniform in structure, amyloid-like fibrils by self-assembly in various solvents, pH's, ionic strengths and temperatures (to be published).

The fibrils were judged to be amyloid-like from their tinctorial and structural characteristics: They bind Congo-red and Thioflavin-T (data not shown). They are straight, unbranched uniform in diameter (~90 Å) double helices of indeterminate length as seen in electron micrographs (Figures 2A, 2B). Each double-helical fibril consists of two protofilaments wound around each other. The protofilaments have a uniform diameter of approximately 30-40 Å. The pitch of the double helix (Figure 2A, arrows) is approximately 920 Å.

Suspensions of these fibrils form oriented fibres, which give characteristic "cross-β" X-ray diffraction patterns (Figure 3). In these fibres the long axes of the amyloid-like fibrils seen in the electron micrographs of Figure 2 are most probably oriented more or less parallel to the fibre axis. This is a generally accepted assumption in such cases (Fraser & McRae, 1973).

The oriented X-ray pattern (Figure 3) taken from these fibres indicates the presence of oriented β-sheets in the amyloid-like fibrils of peptide cA. The presence of reflections corresponding to periodicities of 4.66 and 10.12 Å indicates the existence of β-sheets. The strong meridional reflection at 4.66 Å suggests that the β-sheets are oriented so that their β-strands are perpendicular to the fibre axis (consequently, also to the long axis of
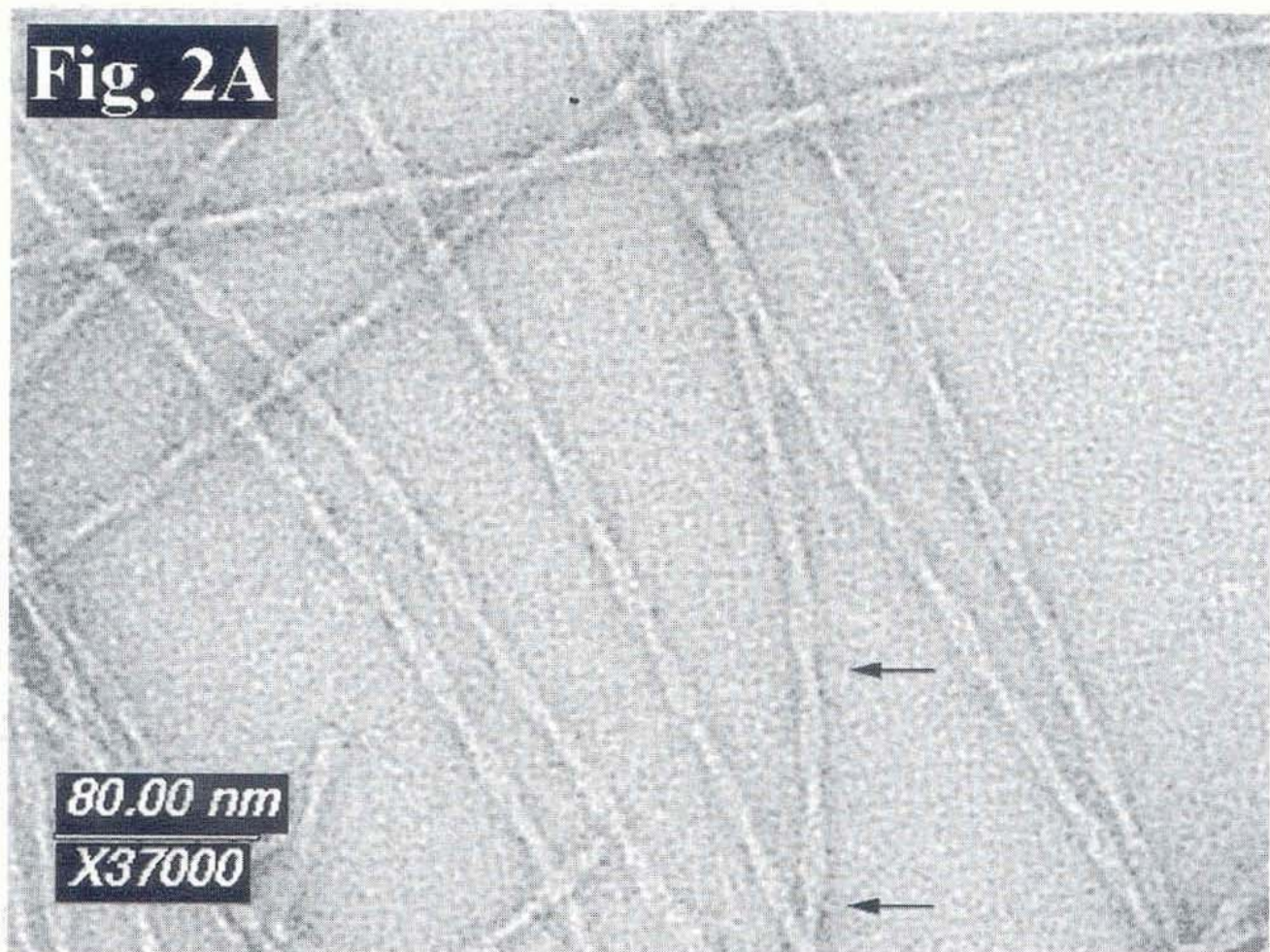
Figure 2A. Electron micrograph of amyloid-like fibrils derived by self-assembly, from a solution 9mg/ml of the cA peptide, in a sodium acetate 50mM buffer, pH 5. Fibrils are negativly stained with 1% uranyl acetate. They are of indeterminate length (several microns), unbranched, approximately 90 Å in diameter and have a double helical structure. The pitch of the double helix is ~ 920 Å (marked with arrows). A pair of protofilaments each 30-40 Å in diameter are wound around each other, forming the double-helical fibrils.

Figure 2B. Electron micrograph of amyloid-like fibrils derived from a solution of the cA peptide (conditions as in Fig. 2A). Fibrils are rotary shadowed with Pt/Pd at an angle of 7 degrees under high vacuum.
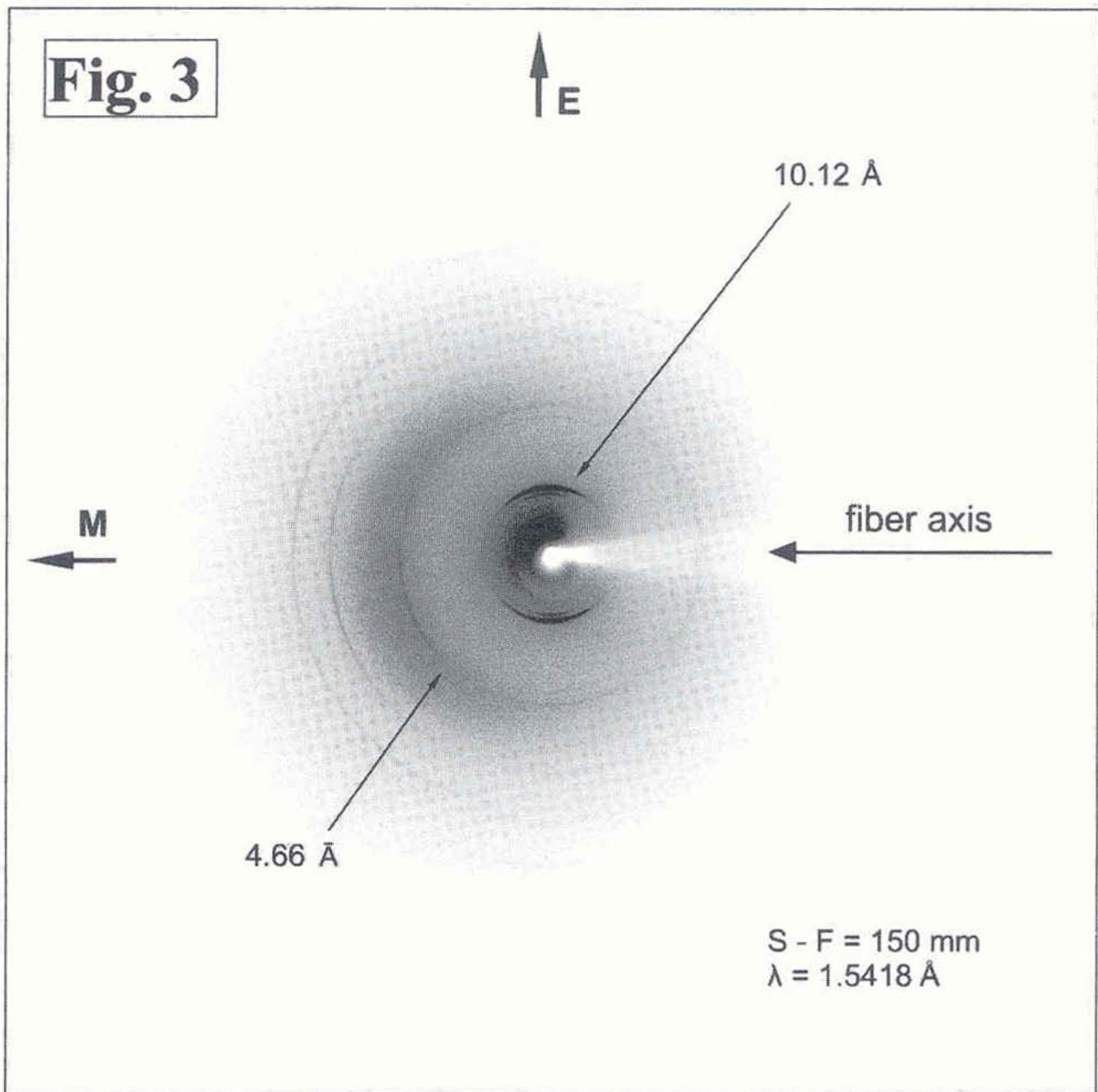
Figure 3. X-ray diffraction pattern from a fibre of cA peptide amyloid-like fibrils. cA peptide was dissolved in a 50 mM sodium acetate buffer pH 5, at a concentration of 9 mg/ml to produce amyloid-like fibrils, after 3-4 weeks incubation. A droplet of fibril suspension was placed between two siliconized glass rods and allowed to dry at room temperature for 1 hr, to form a fibre suitable for X-ray diffraction.

The meridian, M (direction parallel to the fibre axis) is horizontal and the equator, E, is vertical in this display. X-ray diffraction patterns were recorded on a MAR Research 345 image plate utilizing double-mirror focused CuK$_\alpha$ radiation (λ=1.5418 Å), obtained from a GX-21 rotating anode generator operated at 40kV, 75 mA. The specimen to film distance was set at 150 mm and the exposure time was 1 hr.

The X-ray diffraction pattern is a typical "cross-β" pattern showing a 4.66 Å reflection on the meridian and a 10.12 Å reflection on the equator. This indicates a regular structural repeat of 4.66 Å along the fibre axis (meridian) and a structural spacing of 10.12 Å perpendicular to the fibre axis. The structural repeat of 4.66 Å along the fibre axis corresponds to the spacing of adjacent β-strands (which should be perpendicular to the fibre axis, a cross-β structure) and the 10.12 Å spacing perpendicular to the fibre axis corresponds to the face-to-face seperation (packing distance) of the β-sheets.

the amyloid-like fibrils). The strong equatorial reflection at 10.12 Å, which corresponds to the inter-sheet distance, suggests that the packing of the β-sheets is done in a manner parallel to the fibre axis. This X-ray pattern closely resembles typical cross-β patterns taken from amyloid fibres (Sunde & Blake, 1997 and references therein). Therefore, it should arise from a cross-β structure.

The most plausible model that can be proposed for the structure of the cA peptide, to account for all the evidence gathered from this study and from data collected previously on the structure of silkmoth chorion proteins (Hamodrakas, 1992 and references therein), is a model structure shown in Fig. 4.
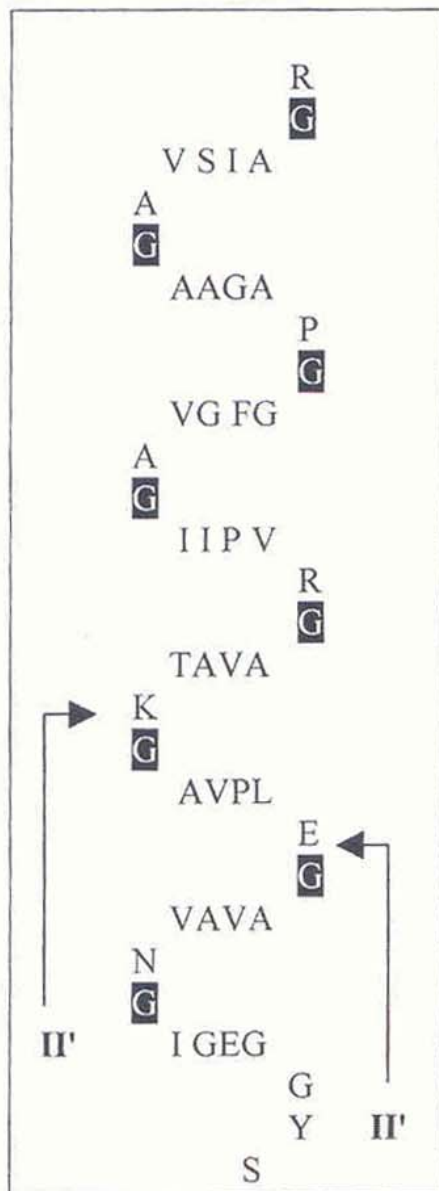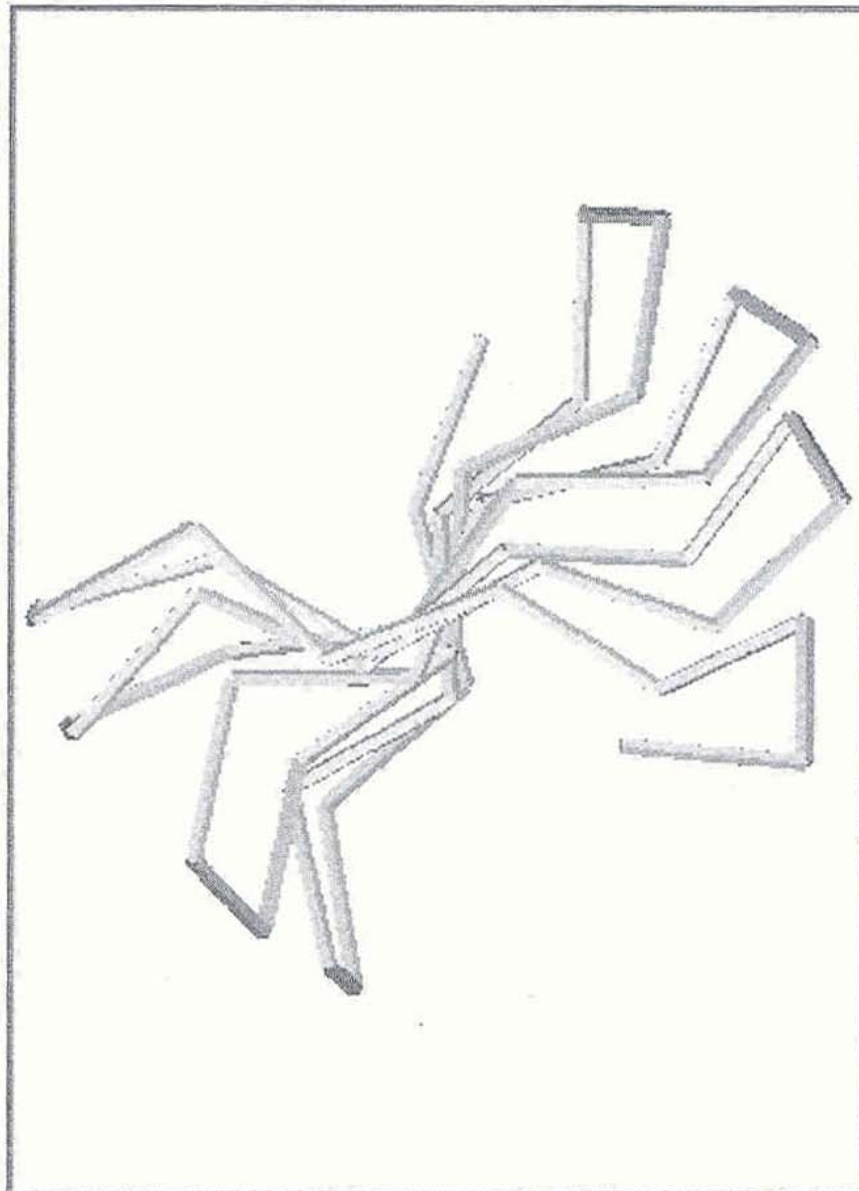

Fig. 4A   **Fig. 4B**

Figure 4A. Antiparallel twisted β-sheet model proposed for the cA peptide. Sequence should be read continuously, beginning at the bottom. Invariant glycines (G) occupying the 2nd position in the β-turns are black boxed. Tentative II' β-turns alternate with four-residue β-strands.

Figure 4B. A skeletal C_a model of the cA peptide showing the characteristic antiparallel β-pleated sheet fold. View perpendicular to the β-strands, parallel to the face of the twisted (helical) sheet.

This model is a slightly modified version of the model proposed previously for the central domain of silkmoth chorion proteins (Hamodrakas et al., 1985; Hamodrakas et al., 1988): An antiparallel twisted β-pleated sheet structure of four-residue β-strands alternating with II' β-turns is proposed to be the structural fold of cA.

The main difference between this model structure and the structure proposed previously (Hamodrakas, 1992 and references therein) is that in the current model, all β-turns are proposed to be II', whereas in the previous model, II' and I' turns alternate along the sequence. The modification of the model was judged to be necessary from the presence of invariant Gly residues in the second position of the β-turns. This is a location especially favourable for Gly in II' turns of twisted β-sheets of globular proteins (Sibanda & Thorton, 1985; Hamodrakas, 1992).

Some 25 years ago, it was proposed that because of the inherent twist of the β-sheets in the monomer, the polymeric protofilaments might form long spacing helical structures in which the protofilaments are intertwined to produce 100 Å doubly helical amyloid fibrils (Cooper, 1976). The verbal description of this model (Cooper, 1976) which is reminiscent of the structure of the β-helix of transthyretin amyloid protofilament produced 20 years later by high-resolution X-ray studies (Blake and Serpell, 1996) fits well to our data.

We propose that successive cA units are forming continuous twisted antiparallel β-sheets (β-sheet helices) along the protofilaments of Fig. 2A, with their β-strands perpendicular to the long axis of the protofilaments (cross-β structures).

The thickness of each individual cA unit is of the order of 30 Å (one has also to consider the effect of the negative stain to fully account for protofilament thickness).

Suspiciously, the pitch of the double helix which is formed by the two intertwined protofilaments is 920 Å (Figure 2A), a multiple of the 115 Å spacing of the β-helix in the transthyretin amyloid protofilament (Blake and Serpell, 1996). Furthermore, the β-sheet model of the cA peptide (Figure 4) is an eight-stranded β-sheet, in contrast to the six-stranded β-sheet of transthyretin. A detailed account of our data analysis will be given elsewhere (Iconomidou & Hamodrakas, in preparation).

Discussion

To our knowledge, this is the first well documented case whereby amyloid-like fibrils are formed from a peptide which has a sequence so clearly folded in an antiparallel β-pleated sheet type of structure, which should be of the cross-β type (the β-strands perpendicular to the long axis of the fibrils). Nature, after millions of years of molecular evolution, has designed these peptides to play an important functional role: to protect the oocyte and the developing embryo from a wide range of environmental hazards (Hamodrakas, 1992). Clearly, amyloids were designed by nature to play a protective role in this case. The fact that the cA peptide, which corresponds to about 30% of the total chorion mass, forming the central domain of silkmoth chorion proteins, produces by self-assembly mechanisms amyloid-like fibrils under a great variety of conditions (Iconomidou & Hamodrakas, In preparation), suggests that it should be folded in an amyloid fashion even in the physiological state. Chorion proteins self

assemble to form the chorion of silkmoths far away from the follicle cells that synthesize and secrete them (Hamodrakas, 1992 and references therein). Experimental and theoretical data support this assumption (Hamodrakas, 1992). However, it might be argued that this is not the only case where amyloids appear *in vivo*. The *Chrysopa flava* silk should be another such case (Geddes et al., 1968).

Another plausible molecular model for the structure of the cA peptide might be that of the left handed parallel β-helix present in the structure of UDP-N-acetylglucosamine acyltransferase (Raetz & Roderick, 1995). The left-handed parallel β-helix is a uniform in cross section structure with a hydrophobic core and a polar coat, composed of a set of three parallel β-sheets forming the three faces of a prism with a cross section that of an equilateral triangle. Each turn of the left handed β-helix consists of a β-strand of four residues followed by two residues in a β-turn (six residues in total), repeated three times, thus forming an equilateral triangle. This is obvious in the aminoacid sequence of the UDP-N-acetylglucosamine acyltransferase, which shows hexapeptide sequence motifs (Raetz & Roderick, 1995). It should perhaps be mentioned that, models similar to the right-handed parallel β-helix found in the pectate lyases were proposed to be the main molecular components of amyloid protofibrils (Lazo & Downing, 1998). Despite the obvious regularities in sequence of the cA peptide, which also shows hexapeptide periodicities both in Gly and in hydrophobic residues (Hamodrakas, 1992 and references therein) and could well fold into a left-handed β-helix (data not shown), there are certain characteristics in the X-ray diffraction patterns and the spectroscopic data (Benaki et al, 1998; Iconomidou & Hamodrakas, In preparation) in clear favour of the antiparallel β-sheet model shown in Figure 4.

A detailed analysis of our model in comparison to the left-handed β-helix model of transthyretin (Blake & Serpell, 1996) which is very similar to ours, will be given elsewhere (Iconomidou & Hamodrakas , In preparation).

Acknowledgements

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