Preliminary Communications

Twisted β-pleated sheet: the molecular conformation which possibly dictates the formation of the helicoidal architecture of several proteinaceous eggshells

Evidence from X-ray diffraction, laser-Raman spectroscopy, secondary structure prediction, freeze-fracturing, conventional electron microscopy and Fourier analysis suggests that the helicoidal structure of the silkmoth eggshell (chorion) is created by protein molecules, most probably in a twisted β-pleated sheet conformation. It is proposed that this conformation also dictates the formation of the helicoidal architecture of other proteinaceous eggshells; apparently, it may also play an important role in the formation of the helicoidal architecture in other biological systems with protein components.

Keywords: Eggshell; chorion; structural protein; helicoidal structure; twisted β-pleated sheet

Several extracellular fibrous structures are known to have helicoidal architecture. Such structures include arthropod cuticles, vertebrate tendons, plant cell walls, etc. (reviewed in Ref. 1). The widespread occurrence of the helicoidal structure in spherical shells, such as eggshells, spore walls, cyst walls and others, and its correlation with the mechanical strength it provides, is intriguing[2].

The helicoidal architecture consists of helicoidally arranged parallel planes, or sheets, of fibrils. Within individual planes, the fibrils are oriented parallel to each other. Between successive planes the fibril direction rotates progressively, thus giving rise to a helix, with its axis perpendicular to the planes[1].

The close analogy between the helicoidal structures of biological materials and the structure of cholesteric liquid crystals probably suggests that several tissues and organelles are self-assembled according to a mechanism that is very similar to the process allowing molecules to form liquid crystals[3]. Therefore, it is important to determine in such cases the molecular mechanisms of self-assembly.

Frequently, as in the case of some insect eggshells, the main components of the helicoidal structure are proteins[4]. It seems interesting to investigate whether a common molecular conformation determines the organization of protein molecules into fibrils and of fibrils into helicoidal structures.

In this preliminary communication, by briefly surveying the evidence on the silkmoth eggshell, or chorion[5-8], and by presenting new data, we propose the twisted β-pleated sheet[9] as the conformation which dictates the formation of the helicoidal structure in proteinaceous eggshells.

The ultrastructure of silkmoth eggshell is relatively complex[10,11]. In the early and middle stages of morphogenesis, it appears to consist of fibrils embedded in a matrix (*Figure 1*). The paraboloidal appearance of these fibrils in oblique sections suggests a helicoidal type of architecture[10,11]. Electron microscopy has shown that these fibrils thicken to the point of confluence during the late period of choriogenesis[4,10]. However, even at this stage, arrays of paraboloidal arcs are discerned. This probably indicates that the helicoidal type of structure is conserved (*Figure 2*).

Freeze–fracturing studies suggest that the helicoidal architecture of silkmoth eggshell is created by fibrils having a helical structure (Hamodrakas, in preparation). It might be expected that these fibrils are formed by some kind of helical substructure. Obviously, possible candidates are the well known α-helix[12], the twisted β-pleated sheet[9], or a collagen type of helix[13], since the components of the silkmoth eggshell fibrils are protein molecules[11].

However, the silkmoth eggshell biochemistry is surprisingly complex: more than 150 different protein components constitute an individual eggshell[14]. These proteins (of relatively low molecular weight) are products of at least three distinct multigene families and are named accordingly as A, B and C[7].

Primary structures have been determined for all three protein classes, either by protein sequencing or by sequencing corresponding cloned DNA sequences (Refs. 5, 7 and 14 and references therein).

Comparisons of these sequences and predictions of secondary structure[5,7] have revealed that all chorion proteins have a tripartite structure: A central domain (*Figure 3*) is highly conserved within each family and is recognizably similar between families; it appears to be highly structured, chiefly forming short β-sheet strands apparently separated by β-turns[5,7]. The flanking amino-

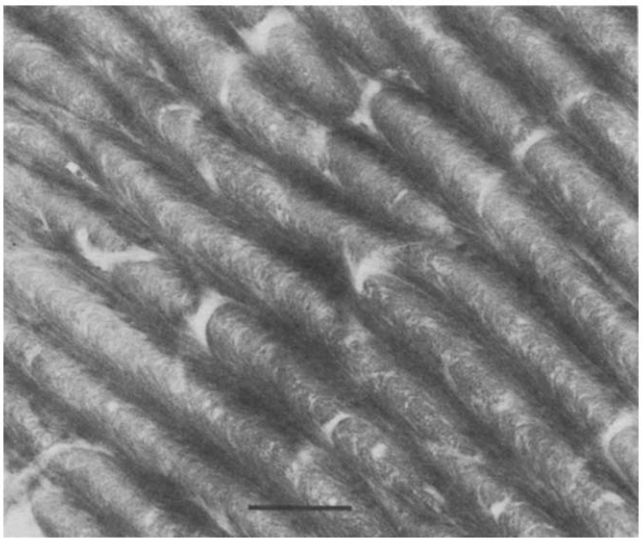

Figure 1 In the middle period of morphogenesis, the main bulk of the silkmoth *Antheraea polyphemus* eggshell (chorion) consists of fibrils, embedded in a matrix. Their paraboloidal appearance suggests a helicoidal type of structure. (Bar is 0.52 μm)

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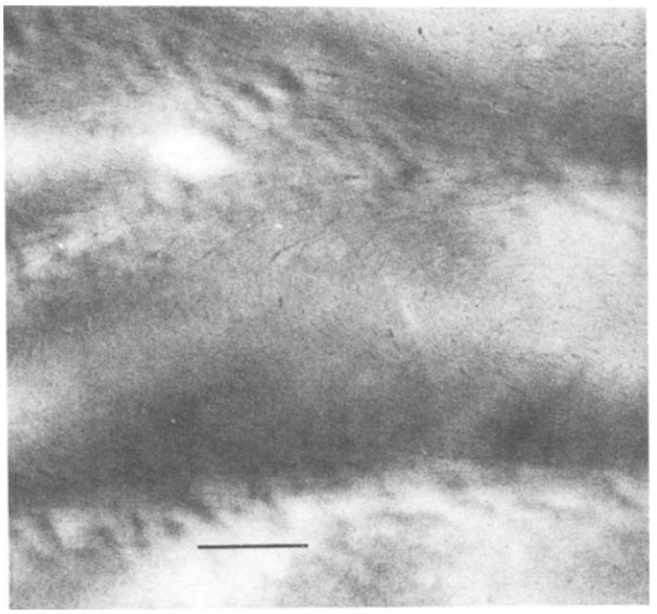

Figure 2 In the late period of choriogenesis, the fibrils coalesce. Even at this stage, paraboloidal arrays of arcs are seen, confirming the helicoidal model of architecture. This micrograph was taken from a thin section of a silkmoth *Bombyx mori* chorion. (Bar is 0.19 μm)

and carboxy-terminal domains ('arms') are more variable and are marked by the presence of tandemly repetitive peptides, directly apparent from the amino acid sequences.

The validity of our structural predictions was tested by laser Raman[6] and X-ray diffraction studies[8]. These studies confirm the preponderance of antiparallel β-pleated sheets in silkmoth eggshell proteins. Furthermore, they indicate a cross β-pleated sheet type of structure[15,6] and suggest a preferred orientation of β-sheets relative to the chorion surface, and complete absence of α-helix or a collagen type of structure[8].

In analogy to the similar systems of feather and scale keratins, it has been assumed that the central, highly conservative and regularly structured domains of silkmoth eggshell proteins are forming the eggshell fibrils, whereas the more variable, in terms of structure and composition, 'arms' constitute the matrix, presumably serving other protein-specific functions (such as crosslinking of fibrils in the late choriogenetic stages, by being particularly enriched in cysteine)[5,7,8].

Recent analysis of the A, B and C classes of silkmoth chorion protein sequences by Fourier methods[16] and methods recently devised (Hamodrakas, Etmektzoglou and Kafatos, in preparation) reveals interesting regularities. In particular, in the abundant A class of proteins, it has been found that the central conservative domain, highly structured into β-sheet strands[5] and probably participating in the formation of chorion fibrils, consists of tandemly repetitive hexapeptides. These hexapeptides can be arranged in an antiparallel β-sheet conformation (schematically shown for one member of the class in *Figure 4*), by noting a six-residue periodicity in β-sheet propensities and a similar, but out of phase, periodicity in β-turn propensities (Hamodrakas, in preparation). The results of the analysis for the other two families of proteins, although similar, are at present less clear.

By combining all the available data, the helicoidal architecture of silkmoth eggshell, the possibly helical structure of its constituent fibrils, the absence of α-helix or a collagen type of structure and the preponderance of antiparallel β-pleated sheets (probably of a regular type) in silkmoth eggshell proteins, we arrive at the plausible proposal that the antiparallel β-sheets, which are presumably constituents of the eggshell fibrils, in turn creating the helicoidal architecture of silkmoth eggshell, are twisted β-sheets[9].

If this hypothesis is correct, the formation of the eggshell fibrils and their self-assembly, which is done extracellularly[4], for the construction of the eggshell helicoidal ultrastructure, should be based on simple stereochemical rules of packing of twisted β-pleated sheets (given in Ref. 17). Our efforts are now concentrated on determining, in detail, these rules for the silkmoth eggshell.

At this point, it is perhaps interesting to note that, at least in one case of formation of cholesteric liquid crystals

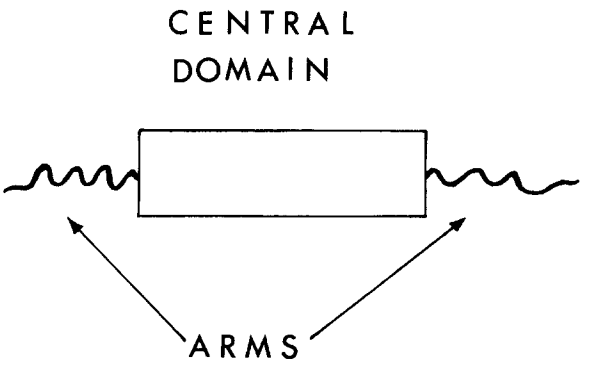

Figure 3 Schematically, the tripartite structure of the silkmoth eggshell proteins. A central, highly conservative and regularly structured domain and two, more variable, flanking 'arms', particularly enriched in cysteine, constitute each protein

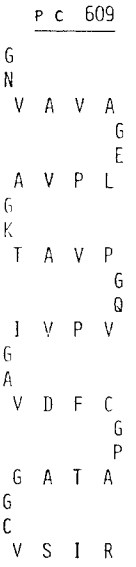

Figure 4 Schematically, the antiparallel β-pleated sheet structure of the central, conservative domain, of the silkmoth eggshell A protein pc 609, which consists of tandemly repetitive hexapeptides

from synthetic polypeptides, the main constituents of the helicoidal structure are α-helices[1-8].

Extending our proposal, we suggest that the helicoidal structure of proteinaceous eggshells might be derived from twisted β-pleated sheets. Further experimental work will show whether our proposal is correct.

S. J. Hamodrakas

Department of Biochemistry, Molecular and Cell Biology and Genetics, University of Athens, Panepistimiopolis, Kouponia, Athens 15 771, Greece

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Preparation and characterization of water-soluble chitin phosphate

New water-soluble chitin derivatives, chitin phosphate of various degrees of substitution, were successfully prepared by the reaction of chitin with phosphorus pentoxide in methanesulphonic acid. These materials behaved hydrodynamically as a typical polyelectrolyte, and showed high ability to adsorb metal ions.

Keywords: Polysaccharides; chitin; chitin phosphate; water-soluble chitin derivatives; polyelectrolyte; adsorption of metal ions

Chitin is known to be abundant in nature. Although this polysaccharide is structurally similar to cellulose, it has poor solubility and is more resistant towards chemical reagents because of its strong micelle structure. The poor solubility of chitin and its derivatives in common solvents has been a major drawback to its utilization. We have prepared chemically modified chitin derivatives which possess good solubility in many kinds of solvents in order to promote the usefulness of this polysaccharide resource. In the course of this project, the acylation reactions in methanesulphonic acid were found to be very efficient, and many kinds of acyl-chitins soluble in organic solvents were successfully prepared by this method[1-4].

Recently, the reaction of chitin with phosphorus pentoxide by this method was found to give water-soluble chitin phosphate (phosphorylated chitin) of high degree of substitution (DS) very efficiently. The preparation of chitin phosphate and its interesting properties, such as the efficient adsorption of heavy-metal ions, have already been reported by Sakaguchi *et al.*[5,6] The most important fact is that its adsorption of uranium was much greater than of other heavy-metal ions[6]. This phenomenon suggests its possible use for the collection of uranium from sea water. However, the DS of chitin phosphate prepared by procedures similar to those for cellulose phosphate[7] was very low. Our novel procedure made it possible to produce a new water-soluble chitin derivative, chitin phosphate of high DS, easily and efficiently. Since our preliminary experiments proved that this material has a high ability to adsorb metal ions, we report here the procedures for its preparation and its rough characterization before performing exhaustive experiments.

Chitin was prepared from Queen Crab shells according to the procedure of Hackman[8], and powdered to 45–60 mesh. To the mixture of chitin (2.0 g) in methanesulphonic acid (14 ml) was added phosphorus pentoxide (0.5–4.0 mol. equiv. to *N*-acetyl-D-glucosamine residue) and the mixture was stirred at 0–5°C for 3 h. The mixture was stood overnight at −20°C, and the product then precipitated with ether. The residue, after washing several times with ether and acetone, was dissolved in deionized water (50 ml) and dialysed completely. A slight amount of insoluble product was removed by filtration, and the filtrate was concentrated *in vacuo* to a small volume. The product, water-soluble chitin phosphate, was precipitated with acetone, collected by centrifugation, washed with acetone and then dried. The yield was 1.0–1.6 g.

The i.r. spectra, ^{1}H and ^{13}C n.m.r. spectra and elemental analyses (C, H, N and P) indicated that these materials were correct chitin phosphates, and further that these chitin derivatives were found to behave viscometrically as a typical polyelectrolyte. The relationship between the amount of phosphorus pentoxide employed and the DS of

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