X-ray diffraction studies of a silkmoth chorion

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High- and low-angle diffraction studies have been performed on mature chorion (eggshell) of the silkmoth, Antheraea polyphemus. *The results confirm the prevalence of β-sheet structure, previously suggested by predictions based on known primary structure and by results of laser Raman spectroscopy. The patterns obtained with different irradiation geometries suggest that a significant proportion of β-sheets are stacked and oriented with respect to the chorion surface and the ultrastructurally evident fibrillar components. Strong similarities are evident with the organization of β-sheets in chicken scale keratin.*

Keywords: Silkmoth chorion; eggshell; β-sheet; X-ray diffraction; structural protein

Introduction

The silkmoth eggshell or chorion is a complex extracellular proteinaceous formation. Its ultrastructure and morphogenesis have been studied extensively[1-6] in parallel with sequencing studies of the component proteins[7-11], and with studies aimed at elucidating the mechanisms that regulate sequential production of these proteins during development[2,12-14].

Biochemically, the chorion is surprisingly complex: as many as 186 protein components have been resolved by two-dimensional gel electrophoresis, from the chorion of eggs produced by an individual *Antheraea polyphemus* moth[3]. At least most of these components are products of distinct structural genes; however, many of these genes are related, i.e. have arisen during evolution by reduplication followed by sequence divergence[7-9,14-15]. Three gene families have been distinguished, A, B and C, corresponding to similarly named size classes of proteins which together account for ~97% of the chorion mass (approximate MW for A proteins 9000 to 12000; for B proteins 12000 to 14000; for C proteins 16000 to 20000).

Primary structures have been determined for all three major protein classes, either by direct protein sequencing[7-9,16] or by sequencing corresponding cloned DNA molecules[11,17-19]. Comparisons of these sequences and predictions of secondary structure[10-11] have revealed that chorion proteins have a tripartite structure. A central domain is highly conserved within each family, and recognizably similar between families; it appears to be highly structured, chiefly forming short β-sheet strands apparently separated by β-turns. The flanking, amino- and carboxy-terminal domains ('arms') are more variable, appear less structured, and are marked by the presence of tandemly repetitive peptides.

The chorion is also very complex in terms of physiology and ultrastructure[6,15]. It consists largely of 10 to 20 nm fibrils (see Discussion for details). The fibrils increase in number during the early and middle periods of morphogenesis, while during the late period they thicken until they coalesce[4]. Both its geometrical structure and the dynamics of its formation suggest that the chorion is a biological analogue of liquid crystals[6]; after its construction, it hardens by formation of disulphide bonds.

With a wealth of compositional and ultrastructural information available, the chorion is a favourable system for studying how structural proteins assemble to form complex, physiologically important structures. In this respect, it is important to relate the known primary and putative secondary structure of the component proteins to the ultrastructurally evident higher order structure. As a first step, we have analysed the structure of mature chorion by X-ray diffraction.

Experimental

Mature and ovulated follicles were dissected from developing female *Antheraea polyphemus* pupae in distilled water, with a crystal of phenylthiourea to inhibit tyrosinase. Follicles were cut in half with fine scissors and washed several times in distilled water to eliminate the oocyte. Swollen follicular cells were peeled off the surface

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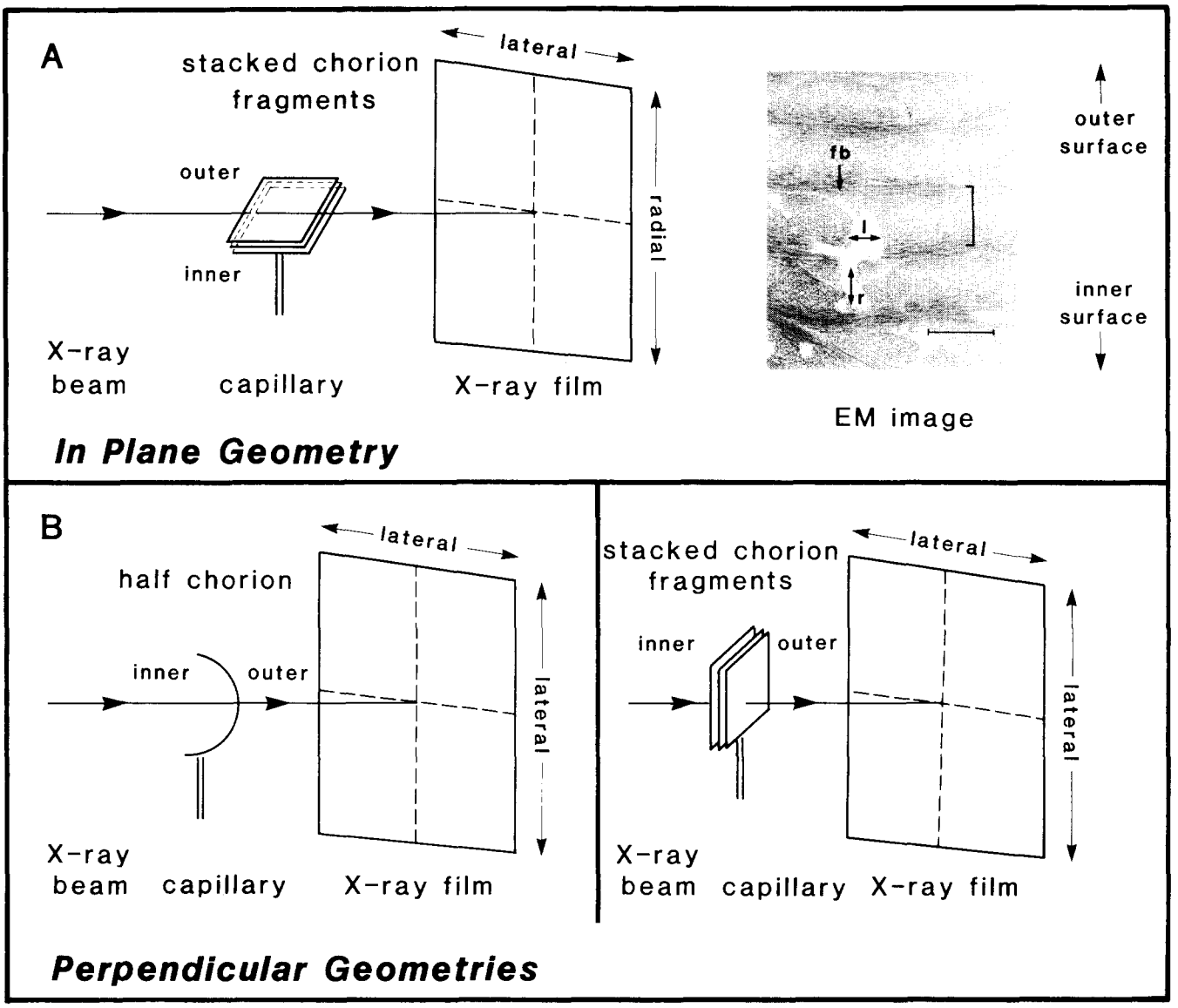

Figure 1 Geometries employed in the high-angle X-ray diffraction experiment. In (a), a stacked array of almost flat fragments, obtained from the hollow chorion sphere, is irradiated with the beam in the plane of the fragments, i.e. parallel to the outer and inner surfaces of the chorion. Note that the horizontal axis of the film is parallel to the fragments and thus reveals lateral periodicities, whereas the vertical axis of the film reveals radial periodicities (along the axis between inner and outer chorion surfaces). The electron micrograph[6] is from an immature chorion and corresponds to the cut face that would be encountered by the beam. Fibrils (fb) are seen in orientations that vary with the plane; the bracket outlines one lamella (180° rotation in fibril orientation), and the lateral (l) and radial (r) axes are indicated, as are the outer and inner chorion surfaces. The bar is 500 nm. In (b), the beam is oriented along the radial axis of the chorion hemisphere, or of stacked chorion fragments; consequently, both the horizontal and the vertical axes of the film reveal lateral periodicities

of the underlying chorion. Insoluble chorions were selected under a dissecting microscope, repetitively washed in 95 and 100% ethanol followed by distilled water to remove the vitelline membrane, and air dried.

Samples used for X-ray diffraction experiments were either hemispherical half-chorions, or stacked arrays of almost flat chorion fragments. High angle X-ray diffraction photographs were taken with the geometries shown in *Figure 1*, using Ni-filtered CuKα radiation obtained from Elliot GX-6 and GX-18 rotating anode generators, running at 30 kV, 40 to 60 mA. Two types of cameras were employed, a camera with Elliot toroidal mirror optics[20] and a camera with Franks optics[21]. Exposure time varied between 3 and 48 h, depending on the camera and the size of the sample used. Dried helium was flushed in the camera during the exposures to eliminate scatter.

To determine the spacings, the X-ray photographs were subjected to densitometry using a computer controlled scanning digital microdensitometer (Optronics P1000). Programs developed at the EMBL, Heidelberg, were used, which employ least squares procedures to refine the scattering centres.

Low angle X-ray diffraction patterns were recorded on a Franks camera with two 20 cm bent-glass mirrors and a 21.2 cm specimen-to-film distance, mounted on a GX-13 rotating anode X-ray generator (Marconi-Elliot Avionics) which produces 0.154 nm Ni-filtered CuKα radiation at 60 mA, 40 kV[22,23]. This camera is capable of a first order resolution of 60 nm or more. The geometry used in the low angle experiments was the one shown in *Figure 1a*.

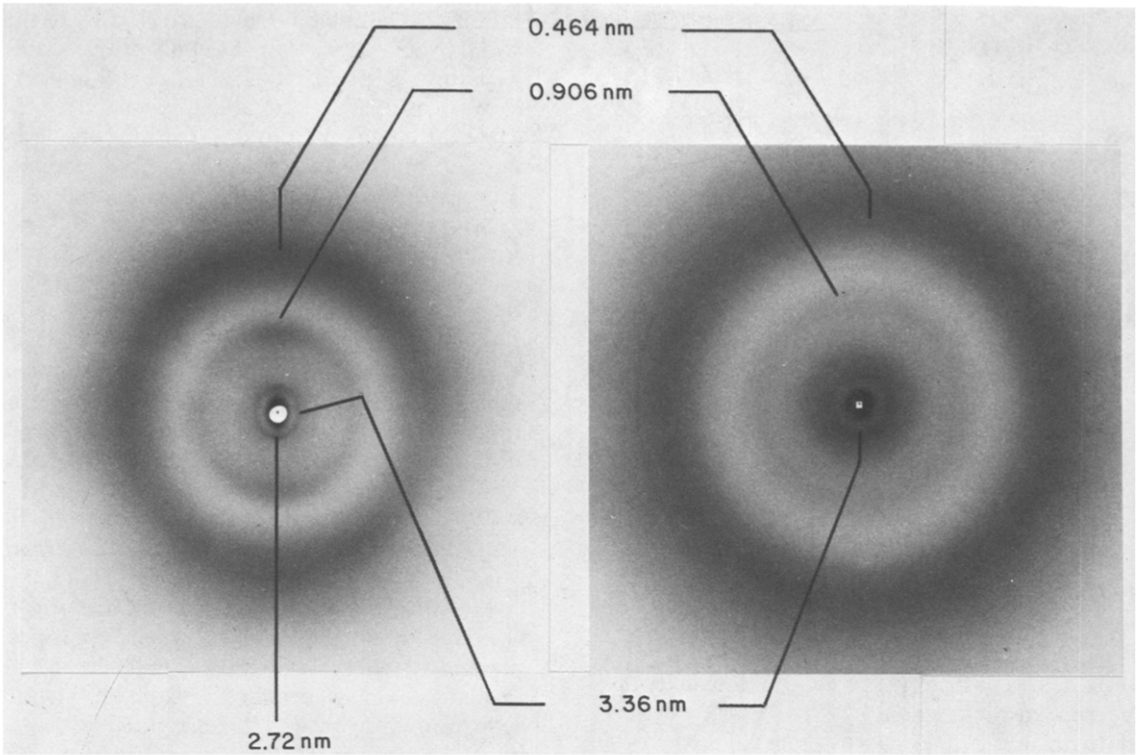

Figure 2 High angle X-ray diffraction patterns from mature silkmoth chorions. Pattern (left) is from the in-plane geometry (cf. *Figure 1a*) and (right) from the perpendicular geometry (cf. *Figure 1b*; half chorion). Note the presence of 0.464 nm, 0.906 nm and 3.36 nm reflections, which form rings in the perpendicular geometry. In the in-plane geometry, the same reflections occur but the 0.906 nm repeat is stronger and predominantly oriented along the radial axis. A 2.72 nm radial repeat is also seen, whereas the 3.36 nm repeat is confined to the lateral axis

Results

High angle X-ray diffraction patterns of silkmoth chorion were obtained using the geometries shown in *Figure 1*. The chorion can be considered as a hollow sphere (it is actually a flattened elipsoid), the inner surface of which faces the oocyte, and the outer surface the environment. In electron micrographs the chorion is seen to be composed of fibrils, which are largely stacked in planes parallel to these two surfaces (see *Figure 1a* and Discussion). Accordingly, in the 'in-plane geometry' (*Figure 1a*), the beam was parallel to the chorion surfaces and to the planes of stacked fibrils. In this case, the vertical axis of the diffraction pattern corresponded to the radial axis of the chorion, i.e. revealed order along the axis from the inner to the outer surface; the horizontal axis of the diffraction pattern corresponded to the lateral axis of the chorion, i.e. revealed order within planes parallel to the surface. In the 'perpendicular geometry' (*Figure 1b*), the beam was perpendicular to both the chorion surfaces and the planes of stacked fibrils. In this case, both the vertical and the horizontal axes of the diffraction pattern revealed lateral order, within these planes.

In both orientations the high angle diffraction patterns had certain features in common (*Figures 2a, 2b*). However, asymmetrical texture was only observed in the in-plane geometry (*Figure 2a*), indicating preferential orientation of the molecular chains.

Both perpendicular and in-plane geometries yielded broad, nearly uniform rings, centred at 0.464 nm. This spacing is typical of β-sheet structures, corresponding to the interchain distance, between hydrogen bonded peptide chains of each sheet (see Discussion).

A second ring was observed, at a 0.906 nm spacing, which may be attributed to the intersheet distance between regularly packed β-sheets (see Discussion). In the perpendicular geometry this ring was uniform and relatively weak (*Figure 2b*). By contrast, in the in-plane geometry it included strong and broad reflections along the radial axis (*Figure 2a*). A third ring corresponded to spacings of ~ 3 nm. In the perpendicular geometry it was circular and corresponded to 3.36 nm. In the in-plane geometry it was oblong, and in some patterns it was clearly resolved into strong 2.72 nm reflections along the radial axis and a weaker 3.36 nm reflection along the lateral axis (*Figure 2a*).

Low angle X-ray diffraction patterns (*Figure 3*) confirmed the existence of oriented 2.72 and 3.36 nm spacings in the in-plane geometry. These patterns revealed no other discrete reflections in the low angle region; diffuse central scatter was seen at very low angles, starting at 8 nm along the radial axis and 11 nm along the lateral axis.

Discussion

Predictions of secondary structure, based on known primary sequences of major chorion proteins, have previously led us to the suggestion that β-sheet structure is predominant in the silkmoth chorion[10,11]. The preponderance of β-sheet structure was also supported by the results of laser Raman spectroscopic studies[24]. The X-ray diffraction studies reported here further support this notion: the prominent 0.464 nm ring may be assigned to the distance between hydrogen bonded antiparallel chains within each sheet, whereas the 0.906 nm ring can be

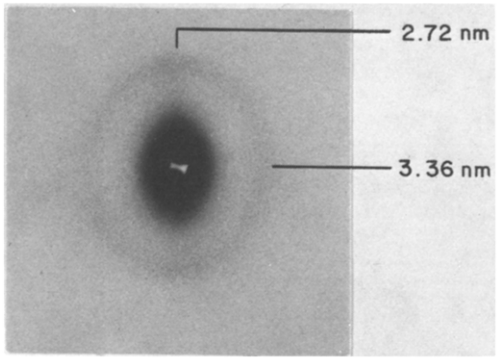

Figure 3 Low angle X-ray diffraction pattern from mature silkmoth chorion, obtained using the in-plane geometry. This pattern confirms the results seen in the low angle region of *Figure 2a*: there is a 2.72 nm radial repeat and a 3.36 nm lateral repeat

attributed to the packing distance between adjacent sheets[25]. By contrast, the X-ray diffraction patterns do not contain a ca. 0.54 nm reflection which would be representative of α-helix, confirming the suggested paucity of α-helical structure in the chorion[10,24].

We wish to draw particular attention to the remarkable similarities between the diffraction patterns presented here, and those previously reported for chicken scale keratin[26]. These similarities include the prominence of interchain and intersheet reflections (0.47 and 0.94 nm, respectively, in the case of scale keratin); the presence of oriented reflections in the in-plane but not the perpendicular geometry; and the presence of oriented reflections in the vicinity of 3 nm, in both high and low angle X-ray diffraction patterns obtained using the in-plane geometry (2, 2.3 and 3.5 nm in the case of scale keratin).

From electron microscopic studies, it is known that the chorion consists of 10 to 20 nm fibrils largely oriented in planes parallel to the chorion surface[1,2,4-6]. Between any two planes, the direction of the fibrils differs by an angle which is proportional to the distance separating the planes. Thus, the chorion fibrils form a helix along a radial axis perpendicular to the plane of the chorion: ~60 lamellae are generated, a lamella being defined by 180° rotation in the direction of the stacked fibrils (for the original description of such 'twisted' models of helicoidally fibrous structures, see Ref. 27). The 60 lamellae belong to four major types, found in corresponding broad layers, from the inner to the outer surface. These four types of lamellae differ in thickness, regularity and presumably composition[3,4,6]. In two broad layers they are well oriented parallel to the surface, as described above, but in a third layer they are disrupted and in a fourth they are oblique to the surface.

When considered together with the electron microscopic evidence, the X-ray diffraction patterns suggest substantial orientation of the β-sheets relative to the fibrils. Since the 0.906 nm reflections are most prominent along the radial axis (in-plane geometry), and weak along the lateral axis (both in-plane and perpendicular geometries), it would appear that β-sheets tend to be stacked across the fibril, occupying planes parallel to the chorion surface (rather than, say, being stacked along the long axis of the fibril). The weak 0.906 nm reflections in the lateral axes could be ascribed either to the disrupted and oblique lamellae, or to stacking of β-sheets in non-radial orientations, even in fibrils which are parallel to the surface. It should be noted that a high proportion of β-sheet structure is predicted for all three major families of chorion proteins, A, B and C, and that in each case β-sheets are predicted not only for the conservative, central domain of the polypeptide chain, but also for part of the more variable, amino- and carboxy-terminal polypeptide regions or 'arms'[10,11]. Regularly stacked β-sheets parallel to the chorion surface may be family-specific, or may represent a polypeptide region-specific subset (e.g. the β-sheet of the central domains), whereas other subsets (e.g. β-sheets of the arms) may be non-stacked, or stacked in various orientations. Non-stacked sheets would explain the paradoxical observation that in the in-plane geometry the 0.464 nm (interchain) reflection is not preferentially observed at a 90° angle relative to the 0.906 nm (intersheet) reflection. Oriented 0.94 nm and unoriented 0.47 nm reflections are also observed in the in-plane patterns derived from scale keratin (see *Figure 2b* in Ref. 26).

Prominent reflections at ~3 nm are also observed in the chorion patterns. In the perpendicular geometry they are unoriented and correspond to 3.36 nm, whereas in the in-plane geometry oriented 3.36 nm and stronger, 2.72 nm reflections are observed. One possible explanation for these reflections is that the fibrils seen by electron microscopy are aggregates of oriented ca. 3 nm filaments. The shape and stacking of these filaments would be asymmetrical in cross-section, resulting in the 2.72 nm radial and 3.36 nm lateral periodicities. Similar filaments, measuring ~3 nm in diameter, and spaced at distances of 3 to 3.5 nm, have been seen in both feather rachis[28] and scale keratin[26]. Since the electron microscopy performed thus far on the chorion has been concerned with elucidation of higher order structure, 3 nm filaments may also exist in the chorion but have escaped detection. An alternative, less likely explanation is that the ca. 3 nm chorion reflections may represent first-order maxima from the thicker fibrils which, at low resolution, can be considered as having continuous electron density.

No periodicities were detected larger than 3.36 nm. It should be noted that in mature chorions, such as those used here, the fibrils have thickened (by addition of ca. 3 nm filaments?) to the point of confluence[6].

In conclusion, this study confirms the predominance of β-sheet structure in the chorion, and reveals a substantial degree of regular orientation in the alignment of β-sheets within the fibrillar substructure. Further X-ray diffraction and high resolution electron microscopic studies are necessary to relate these features to the supramolecular organization of the chorion. Such studies should be undertaken both with chorions at various morphogenetic stages, and with fibres or films of purified chorion proteins and protein classes.

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