Fine structure of the silkmoth *Antheraea polyphemus* chorion as revealed by X-ray diffraction and freeze-fracturing

S. J. Hamodrakas, L. H. Margaritis, I. Papassideri
Department of Biochemistry, Cell and Molecular Biology and Genetics, University of Athens, Panepistimiopolis, Kouponia, Athens 157.01, Greece

and A. Fowler
European Molecular Biology Laboratory, Meyerhofstrasse 1, PO Box 10.2209, 6900 Heidelberg, FRG
(Received 19 November 1985; revised 13 January 1986)

The filamentous organization of silkmoth chorion has been investigated, by studying the effect on the X-ray patterns, of reaction with heavy atoms. Considerable variation in the intensity of the low angle reflections was observed after deposition of osmium and uranium in the structure and the possible significance of this observation is discussed in relation to the organization of the fibrous structure of chorion. In addition, application of the technique of freeze-fracturing, with single-sided and rotary shadowing, reveals that silkmoth chorion consists of filaments, approximately 3 nm in diameter, and provides a direct visualization of the helicoidal arrangement of these filaments for the formation of chorion architecture. The packing of the filaments is in good agreement with the X-ray data. There are indications that the filaments have a helical structure.

Keywords: Silkmoth; eggshell; chorion; X-ray diffraction; freeze-fracturing

Introduction

The silkmoth chorion, the major component of the eggshell, is a proteinaceous, protective and functional layer surrounding the oocyte. We have chosen the chorion of the silkmoth as a model system to study how proteins self-assemble to form complex, physiologically important structures[1-7].

Chorion proteins are predominantly organized as fibres embedded in a matrix[8,9], which suggests analogies of chorion with vertebrate keratins and other fibre-matrix systems (Refs 1–7 and references therein). The helicoidal orientation of these fibres indicates similarities with arthropod cuticle and many other systems of structural proteins[10,11]. The chorion consists of fibrous layers parallel to the chorion surface. Between adjacent layers the direction of the fibres differs by a constant angle, resulting in a helicoidal structure (cf. Ref. 11) which is a biological analogue of a cholesteric liquid crystal[12]. The structure changes dramatically during morphogenesis and also varies locally, consistent with the biochemical complexity and the multiple physiological functions of the eggshell[13].

Recently, we proposed the antiparallel twisted β-pleated sheet as the molecular conformation which dictates the formation of the helicoidal architecture of silkmoth chorion[4,7]. This proposal was based on evidence from X-ray diffraction, laser-Raman and infrared spectroscopy, transmission electron microscopy, secondary structure prediction and Fourier analysis of the known amino acid sequences of the A, B and C classes of chorion proteins, which are products of distinct, but related multigene families (Refs. 1–7 and references therein).

Electron microscopy has shown that, in the middle choriogenetic stages, the chorion consists of fibrils, 10–20 nm in diameter, embedded in a matrix[1,2], which thicken until the coalesce as chorion matures. However, in the final choriogenetic stages it was not possible, until recently, to discern individual fibrils with conventional transmission electron microscopy[14]. On the other hand, X-ray diffraction studies have shown that in mature (ovulated) chorions 3 nm periodicities exist[3]. These periodicities were attributed to packing distances between fibrillar elements, named filaments to distinguish them from the 10–20 nm fibrils appearing in the middle stages of choriogenesis. Whether these filaments actually exist and what their relation is to the 10–20 nm fibrils remained to be elucidated.

In this report we present data from X-ray diffraction experiments and freeze-fracturing studies, which show conclusively that, in mature chorion, filaments with a diameter of approximately 3 nm actually exist and provide a direct experimental visualization of the helicoidal structure of chorion. This information may prove useful for the achievement of our ultimate goal, that is to relate the primary sequences and tripartite composition of chorion proteins to the fibrous structures they assume and the regular assemblies of these fibres, namely the chorion.

Methods

Preparation of purified chorions

Mature and ovulated follicles were dissected from female *Antheraea polyphemus* pupae in distilled water, with a crystal of phenylthiourea to inhibit tyrosinase. Follicles

0141-8130/86/040237-06$03.00

were cut in half with fine scissors and washed several times in distilled water. Swollen epithelial cells were peeled off the surface 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.

X-ray diffraction

Samples used for X-ray diffraction were chorion fragments. X-ray diffraction patterns were taken with the beam either parallel (in-plane geometry) or perpendicular (perpendicular geometry) to the surface of the chorion[3]. Ni-filtered CuKα radiation was used ($\lambda = 1.54$ Å), obtained from an Elliot GX-18 rotating anode generator, running at 40 kV, 30–40 mA. A camera with Elliot toroidal mirror optics was employed and exposure times varied between 4 and 72 h, depending on the sample. Dried helium was flushed in the camera during the exposures to reduce air-scatter.

For some experiments a camera with Franks optics was also used. Before X-ray irradiation, some samples were incubated for 12 h in a urea buffer (6 M urea, 0.3 M Tris–HCl, 1 mM glycine, 1 mM Na EDTA, pH 8.5). thereafter referred to as UB. Others were treated with 2% OsO_4 for 60 min at 4°C, and a third category of samples with 2% uranyl acetate for 60 min.

Transmission electron microscopy

Transmission electron microscopy was performed as described elsewhere[6].

Freeze-fracture electron microscopy

Purified half chorions were frozen on golden hats in Freon-12 and liquid nitrogen. Prior to freezing, the samples were incubated for 12 h in the UB (see above), fixed in 2.5% glutaraldehyde for 3 h. Freeze-fracturing was performed in a Balzers 500 unit, modified for rotary shadowing[15]. Some samples were also single-sided shadowed.

Great difficulty was experienced in attempts to fracture silkmoth chorion samples. Fracturing, at −125°C, was made possible only after extraction of the samples with the UB which 'softens' the chorion.

Metal evaporation was performed with electron bombardment guns using Pt-C electrodes. The guns were set at a 35° angle to the specimen table surface, both for single-sided and for rotary shadowing. The replicas were cleaned with sodium hypochlorite for 6 h, followed by chromic acid overnight and, after distilled water washing, they were picked up on 400-mesh electron microscope grids.

Electron microscopy was performed with Phillips EM200 and 301 microscopes, operating at 60 or 80 kV.

All electron micrographs for freeze-fracturing are positive images, i.e. platinum deposits appear dark.

Results

In order to appraise the changes in chorion ultrastructure after urea extraction (see Methods), thin sections of urea extracted chorions were examined by conventional transmission electron microscopy. A typical thin section is shown in *Figure 1*. X-ray diffraction patterns of the silkmoth *Antheraea polyphemus* chorion are presented in *Figure 2*: The pattern of *Figure 2a* was obtained from a purified, almost flat, chorion fragment using the in-plane geometry. It is similar to the patterns obtained by Hamodrakas *et al.*[3] and it is used here as a reference pattern. *Figure 2b* shows the diffractogram obtained after the treatment of chorion with 2% uranyl acetate for 60 min before irradiation.

As noted previously[3], the diffraction patterns of the silkmoth chorion are relatively poor and they are dominated by the presence of certain characteristic reflections: A broad, nearly uniform ring at 1/0.46 nm^{-1} indicating the presence of β-sheet structure in chorion proteins (the abundance of β-sheet structure has also been verified by the use of other experimental methods; see for example Refs. 2 and 6), a reflection at 1/0.9 nm^{-1}, approximately, probably corresponding to the intersheet packing distance, and meridional and equatorial reflections at approximately 1/3 nm^{-1}, which were considered as due to packing distances between fibrillar elements termed filaments, presumably existing in chorion[12]. Differences in the X-ray patterns obtained from the in-plane and perpendicular geometries are discussed in some detail in the same work.

The diffraction patterns obtained after the treatment of chorion with various agents (*Figure 2b* and data not shown) retain the features of those obtained from native chorions (*Figure 2a*). However, pronounced differences are observed in the intensities of certain reflections, depending on the treatment of the samples prior to X-ray irradiation. Thus, a dramatic increase in the intensity of the 1/3 nm^{-1} reflections is seen, when the chorions are treated with osmium tetroxide (data not shown) or uranyl acetate (*Figure 2b*) before the exposure, whereas the same reflection becomes more diffuse after the treatment of chorion with the urea buffer (data not shown).

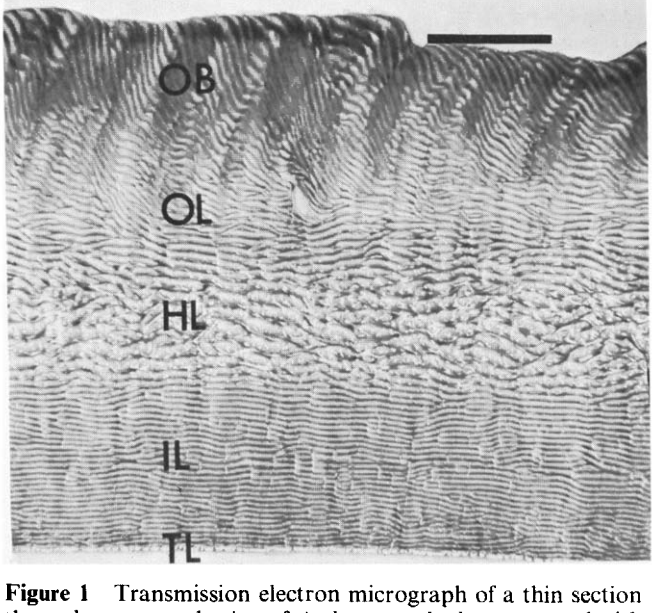

Figure 1 Transmission electron micrograph of a thin section through a mature chorion of *Antheraea polyphemus* treated with a urea buffer (see Methods), before fixation. Within the bulk of the lamellar chorion, four types of lamellae can be distinguished: thin lamellae of the inner lamellar layer (IL), thick, distorted, spongy lamellae of the holey layer (HL), lamellae of the outer layer (OL) and, lying at an angle to the rest, the thick lamellae of the oblique layer (OB). Nearest to the oocyte is the trabecular (TL) layer which consists of pillars surrounding air-filled spaces. × 1680; bar is 10 μm

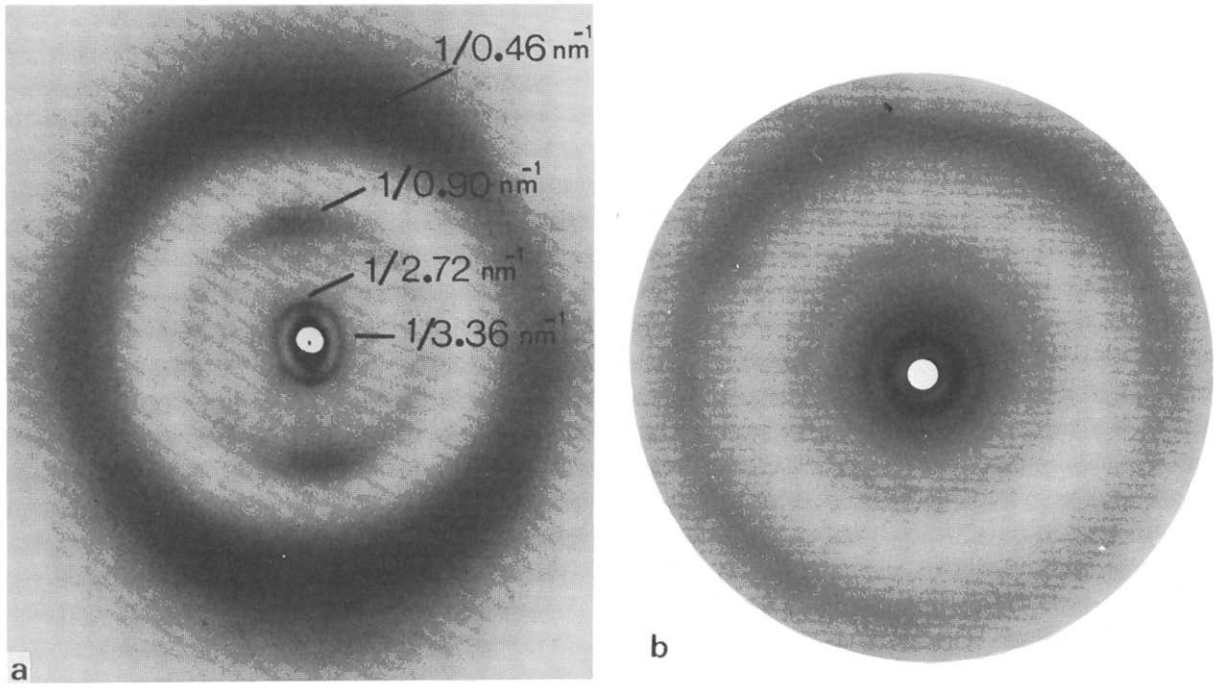

Figure 2 X-ray diffraction patterns from mature silkmoth chorions of *Antheraea polyphemus*. (a) Incident beam parallel to the chorion surface which is horizontal (in-plane geometry). The plane of the film is vertical. (b) Geometry as in (a). The chorion sample was treated with 2% uranyl acetate for 60 min before irradiation. To obtain (a) and (b) a toroidal camera was employed. Specimen to film distance, approximately 70 mm

In addition, two other reflections are seen on the patterns obtained after the 'staining' of chorion with uranyl acetate. They appear at $1/1.8\,nm^{-1}$ and $1/1.25\,nm^{-1}$ approximately. In the OsO_4 pattern only one reflection appears at $1/1.8\,nm^{-1}$. The implications of the variations in the intensity of the $1/3\,nm^{-1}$ reflection and the presence of one or two additional reflections in the diffraction patterns of the 'stained' chorions are discussed below.

A view of a part of the *Antheraea polyphemus* chorion after freeze-fracturing and single-sided shadowing is shown in *Figure 3*, whereas *Figure 4* presents a view of the chorion after freeze-fracturing and rotary shadowing. These micrographs show clearly the fibrillar nature and the lamellar ultrastructure of silkmoth chorion.

Discussion

In this study we focused our efforts in attempts to identify the basic structural elements of chorion and their packing modes in the final choriogenetic stages.

Two lines of approach were followed: In the first, we tried with simple X-ray diffraction experiments to show that the low angle periodicities of approximately 3 nm that appear in the X-ray patterns are due to packing distances between fibrous elements of chorion structure which, therefore, should have dimensions of this order of magnitude. In the second, we attempted to observe directly, by freeze-fracturing, details of chorion ultrastructure at a resolution of 2–2.5 nm; this is the resolution of the technique[15]. Both variations of the technique were applied: single-sided (unidirectional) and rotary shadowing.

In order to be able to apply the freeze-fracturing technique in *Antheraea polyphemus* chorions it was found necessary to 'soften' the mature chorions by treating them with a urea buffer (UB). Since the effect of the urea buffer to the ultrastructure of chorion was unknown, chorions subjected to urea extraction were compared with native chorions in terms of structure (compare *Figure 1* with Figure 1a of Regier *et al.*[16]). The comparison shows that the gross structural features of chorion remain almost unaltered after the urea extraction, which probably indicates that the basic structural units of chorion remain almost intact, with the concomitant conservation of chorion ultrastructure.

To examine whether the $1/3\,nm^{-1}$ reflections, appearing in the X-ray diagrams, correspond to periodicities between fibrous elements of chorion a simple experiment was carried out, similar to that of Fraser and McRae[17] in studies of feather keratin: The silkmoth chorion was treated with 2% osmium tetroxide and 2% uranyl acetate, which results in considerable deposition of osmium and uranium within the structure, as evidenced by the brown and yellow coloration, produced respectively. The high angle diffraction pattern is unaffected by these treatments and it may be concluded, as in feather keratin[17], that deposition of osmium and uranium occurs mainly between, rather than within, the fibrous elements of the structure. At low angles, the principal effect is a dramatic intensification of the $1/3\,nm^{-1}$ reflection, which indicates periodic fluctuations

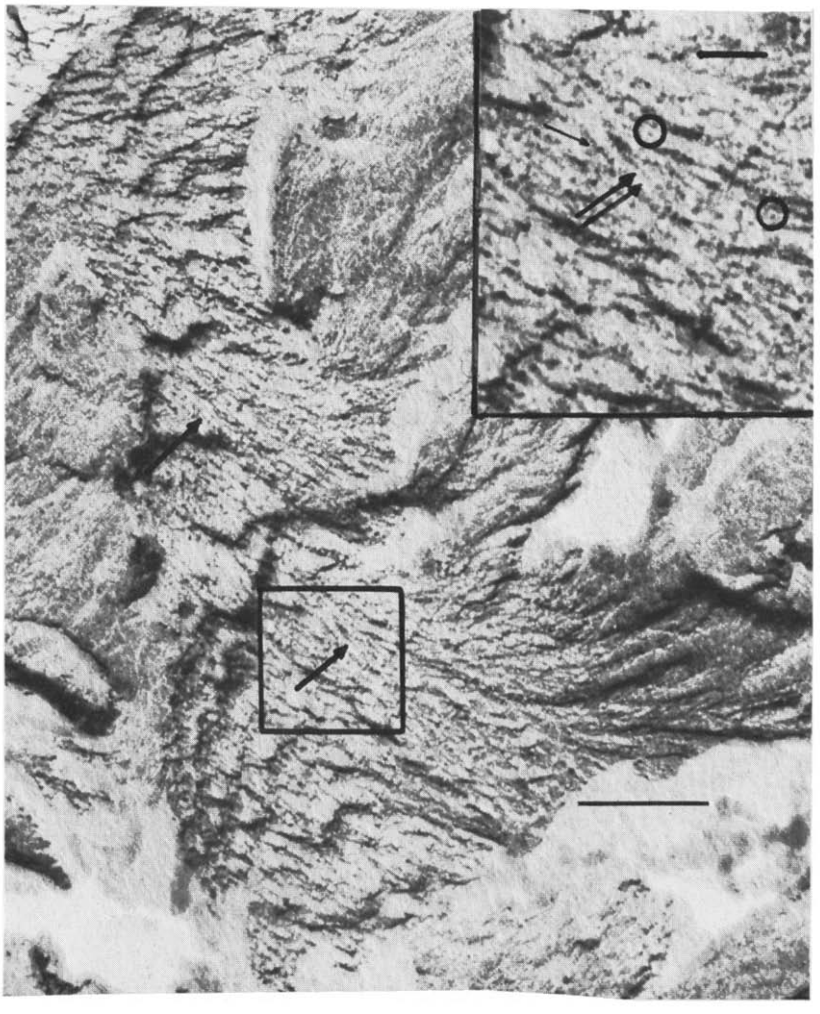

Figure 3 Pt/C replica of a freeze-fracture plane within the chorion of *Antheraea polyphemus* (unidirectional shadowing). The replica shows clearly the fibrillar nature of chorion (arrows). At higher magnification (inset), filaments of diameter approximately 3–4 nm are longitudinally (arrow) or transversely (circles) seen. There are indications (multiple arrows) that these filaments have helical structure. × 85000, bar is 0.2 μm. Inset: × 153000, bar is 60 nm

of considerable amplitude in the density of osmium and uranium in the structure, of the same order of magnitude.

If the $1/3\,nm^{-1}$ reflection is considered as indicating packing distances between chorion filaments, then the dramatic increase in the intensity of this reflection may be easily explained, since the contrast between the filaments and the matrix is considerably increased, if osmium tetroxide and uranyl acetate bind to the matrix intervening between the filaments. The concept that osmium tetroxide and uranyl acetate bind preferentially to the matrix is supported by the proposal put forward by Hamodrakas *et al.* (Ref. 1 and references therein), that the 'less structured', high in cysteine content, variable arms of chorion proteins constitute the matrix. The high affinity of osmium tetroxide and uranyl acetate to cysteine is well known. However, an alternative explanation is that the filaments constituting chorion are formed by the hydrophobic cores of the folded proteins and that the matrix corresponds to the hydrophilic exterior of the proteins, with osmium tetroxide and uranyl acetate binding to the polar groups of the protein surface; groups of the protein surface include the side chains of glutamate, aspartate and cysteinyl residues[4] to which uranyl acetate and osmium tetroxide preferentially attach[18].

The fact that the $1/3\,nm^{-1}$ reflection becomes more diffuse after urea extraction whereas the high angle pattern remains almost unaffected can be explained by assuming that the denaturing agent urea only slightly disorganizes the packing of the disulphide cross-linked proteins, leaving their secondary structure almost intact.

The appearance of the extra reflections, after heavy atom 'staining', emphasizes that, indeed, there is an increase of contrast caused by the binding of osmium tetroxide and uranyl acetate to the chorion proteins.

The striking parabolic patterning of the fibrils which constitute chorion in the middle stages of choriogenesis, as seen in any oblique thin section, has led to the proposal[8] that the chorion of the silkmoths is formed according to the theory given in detail by Bouligand[11], to explain the occurrence of parabolic figures in sections of various, widely diverse, biological systems. Direct visualization of the helicoidal architecture for silkmoth chorion had not been given so far (although this is not true for other helicoidal structures, see for example Refs. 19 and 30). In *Figures 3* and *4*, which are views of parts of the structure of chorion, obtained by using the technique of freeze-fracturing with single-sided (*Figure 3*) and rotary (*Figure 4*) shadowing, a direct confirmation of Smith *et al.*'s proposal is given.

The fibrillar nature of chorion and the organization of

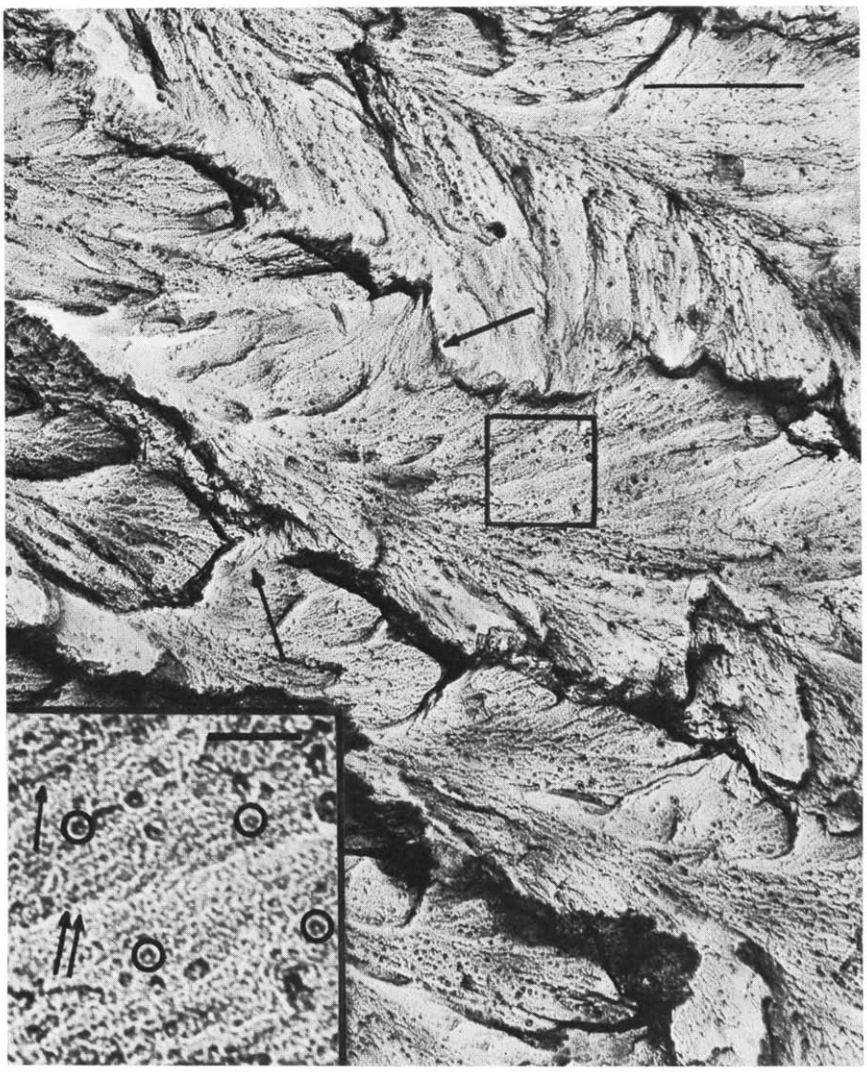

Figure 4 Pt/C replica of a freeze-fracture plane within the chorion of *Antheraea polyphemus* (rotary shadowing). The fracture has advanced across successive lamellae producing a series of steps. Therefore, the lamellar structure of silkmoth chorion is clearly seen. The helicoidal arrangement of chorion filaments is also obvious (arrows). In the inset (boxed area), at higher magnification, the helical (?) (multiple arrows) chorion filaments are seen either longitudinally (arrows) or transversely (circles). × 70 000, bar is 0.3 μm. Inset: × 250 000, bar is 50 nm

chorion fibrous elements are directly evident from *Figures 3* and *4*. However, it appears that these fibrous elements, thereafter named filaments, are thinner than the 10–20 nm fibrils observed early in choriogenesis with transmission electron microscopy[1,2]. Their cross-sections seem to have dimensions of the order of 3 nm, as seen in *Figures 3* and *4* observing them either longitudinally (*Figures 3* and *4* and insets, arrows), or transversely (*Figures 3* and *4*, circles). Their packing arrangement seen in *Figure 4* (inset) results in periodicities of the order of 3 nm, in agreement with the values obtained from the X-ray diffraction patterns.

It is interesting to note that in the fibre-matrix systems of feather and scale keratins, systems analogous in many respects to silkmoth chorion in terms of structure of their component proteins at the primary and secondary levels[1-3] and ultrastructure[1,2], the fibrous units of structure have diameters of the order of 3 nm and are embedded in an amorphous matrix[20,21]. Also, in other systems of structural proteins composed mainly of β-sheets[22-24], the basic units of structure, have dimensions of the order of 3 nm. Therefore, it appears that fibrous units with dimensions of this order are very common in fibrous proteinaceous structures composed of β-sheets. It is worth noting that several β-sheets in globular proteins have also dimensions of approximately 3 nm [25].

We should also mention that in the models that we propose for the structure of chorion proteins[4], the cross-sections of the protein molecules have diameters of the same order of magnitude. Therefore, it would appear that a single (or perhaps a pair of) protein molecule(s) may correspond to a single chorion filament.

The lamellar ultrastructure of chorion is seen in *Figure 4*. It is obvious that the filament direction changes in a helicoidal manner (arrows), in agreement with Bouligand's model.

It is tempting to speculate on the helicoidal assembly process, since it is the basis for the formation of such outstanding (in terms of properties) structure as is the silkmouth chorion: It has been suggested that chorion architecture has a self-assembling, liquid crystalline origin of rod-like components[8]. Indeed, the follicle (epithelial) cells secreting chorion do not play a direct part in the organization of the helicoidal architecture[8], therefore, it is certain that the helicoidal arrangement of the filaments is

due to the molecular organization and packing of their constituent proteins.

Inspired by the paper of Rudall[26], who proposed that a helicoidal structure can be generated from helical basic units, having observed that chorion filaments have a beaded appearance which may be attributed to a helical structure[27], knowing that a characteristic antiparallel β-pleated sheet structure is the secondary structure of chorion proteins (Ref. 4 and references therein) and that β-sheets are usually twisted β-sheets[25], we recently proposed the twisted β-pleated sheet, a helical structure, as the molecular conformation which dictates the helicoidal architecture of silkmoth chorion[7].

In the same report we suggested that the self-assembly procedure should be based on simple stereochemical rules of packing of twisted β-pleated sheets. In this context, it is interesting to note that recently helically twisted, lamellar single crystals of *Bombyx mori* silk fibroin were observed[28], a fibrous protein chiefly in β-sheet structure. These were considered to arise because of the twist of the β-sheets forming the structure.

It is obvious that the helicoidal architecture of silkmoth chorion is well suited for rendering chorion its mechanical properties, mainly strength, which is necessary for its protective function[8,29,31].

In conclusion, in this study, by modifying the X-ray patterns of silkmoth chorion, introducing heavy atoms to its structure, and by applying the technique of freeze-fracturing we verify its helicoidal architecture and we identify with certainty its basic structural units. Further work is needed at well defined developmental stages to understand fully the morphogenesis and subtle secrets of silkmoth chorion structure.

Acknowledgements

S. J. H. thanks the Greek Ministry of Research and Technology and the University of Athens for financial support, the EMBL, Heidelberg for hospitality and acknowledges the help of an EMBO short-term fellowship.

References

1 Hamodrakas, S. J., Jones, C. W. and Kafatos, F. C. *Biochim. Biophys. Acta* 1982, **700**, 42
2 Hamodrakas, S. J., Asher, S. A., Mazur, G. D., Regier, J. C. and Kafatos, F. C. *Biochim. Biophys. Acta* 1982, **703**, 216
3 Hamodrakas, S. J., Paulson, J. R., Rodakis, G. C. and Kafatos, F. C. *Int. J. Biol. Macromol.* 1983, **5**, 149
4 Hamodrakas, S. J., Etmektzoglou, T. and Kafatos, F. C. *J. Mol. Biol.* 1985, **186**, 583
5 Regier, J. C., Kafatos, F. C. and Hamodrakas, S. J. *Proc. Natl. Acad. Sci. USA* 1983, **80**, 1043
6 Hamodrakas, S. J., Kamitsos, E. I. and Papanicolaou, A. *Int. J. Biol. Macromol.* 1984, **6**, 333
7 Hamodrakas, S. J. *Int. J. Biol. Macromol.* 1984, **6**, 51
8 Smith, D. S., Telfer, W. H. and Neville, A. C. *Tissue Cell* 1971, **3**(3), 477
9 Kafatos, F. C., Regier, J. C., Mazur, G. D. Nadel, M. R., Blau, H. M., Petri, W. H., Wyman, A. R., Gelinas, R. E., Moore, P. B., Paul, M., Efstratiadis, A., Vournakis, J. N., Goldsmith, M. R., Hunsley, J. R., Baker, B., Nardi, J. and Koehler, M. in 'Results and Problems in Cell Differentiation', Springer-Verlag, New York, Heidelberg and Berlin, 1977, Vol. 8, 45–145
10 Neville, A. C. in 'The Biology of the Arthropod Cuticle', Springer-Verlag, New York, Heidelberg and Berlin, 1975
11 Bouligand, Y. *Tissue Cell* 1972, **4**(2), 189
12 Mazur, G. D., Regier, J. C. and Kafatos, F. C. in 'Insect Ultrastructure', (Eds. H. Akai and R. C. King), Plenum Press, New York, 1982, p. 150
13 Kafatos, F. C. in 'Gene Structure and Regulation in Development', 41st Symp. of the Soc. for Develop. Biol. (Eds. S. Subtelny and F. C. Kafatos), Alan Liss Inc., New York, 1983, pp. 43–61
14 Papanicolaou, A., Margaritis, L. H. and Hamodrakas, S. J. *Can. J. Zool.* 1984, in press
15 Margaritis, L. H., Elgsaeter, A. and Branton, D. *J. Cell Biol.* 1977, **72**, 47
16 Regier, J. C., Mazur, G. D., Kafatos, F. C. and Paul, M. *Dev. Biol.* 1982, **92**, 159
17 Fraser, R. D. B. and McRae, T. P. *J. Mol. Biol.* 1959, **1**, 387
18 Blundell, T. L. and Johnson, L. N. in 'Protein Crystallography', Academic Press, New York, London and San Francisco, 1976
19 Gubb, D. *Tissue Cell* 1975, **7**(3)(1), 19
20 Filshie, B. K. and Rogers, G. E. *J. Cell Biol.* 1962, **13**, 1
21 Stewart, M. *J. Ultrastruct. Res.* 1977, **60**, 27
22 Geddes, A. J., Parker, K. D., Atkins, E. D. T. and Beighton, E. *J. Mol. Biol.* 1968, **32**, 343
23 Dobb, M. G., Fraser, R. D. B. and McRae, T. P. *J. Cell Biol.* 1967, **32**, 289
24 Burke, M. J. and Rougvie, M. A. *Biochemistry* 1972, **11**(13), 2435
25 Schulz, G. E. and Schirmer, R. H. in 'Principles of Protein Structure', Springer-Verlag, New York, Heidelberg and Berlin, 1978
26 Rudall, K. M. in 'Lectures on the Scientific Basis of Medicine', Athlone Press, London, 1956, Vol. 5, p. 217
27 Manley, R. St. J. *Nature* 1964, **204**, 1155
28 Lotz, B., Gouthier-Vassal, A., Brack, A. Magoshi, J. *J. Mol. Biol.* 1982, **156**, 345
29 Grierson, J. P. and Neville, A. C. *Tissue Cell* 1981, **13**(4), 819
30 Giraud-Guille, M. M. *Tissue Cell* 1984, **16**(1), 75
31 Margaritis, L. H. in 'Comprehensive Insect Physiology, Biochemistry and Pharmacology', (Eds. G. A. Kercut and L. I. Bilbert), Pergamon Press, Oxford, 1985, Vol. 1, pp. 153–230