Three-dimensional reconstruction of innermost chorion layer of *Drosophila grimshawi* and *Drosophila melanogaster* eggshell mutant *fs(1)384*

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A low-resolution three-dimensional structure of the crystalline innermost chorionic layer (ICL) of the Hawaiian species Drosophila grimshawi *and the* Drosophila melanogaster *eggshell mutant* fs(1)384 *has been calculated from electron microscope images of tilted negatively stained specimens. The isolated ICL of* Drosophila grimshawi *is a three-layer structure, about 36 nm thick, whereas the ICL of* Drosophila melanogaster *eggshell mutant* fs(1)384 *is a single layer, about 12 nm thick. Each unit cell in both crystalline structures includes octamers made up of four heterodimers. Crosslinks between the structural elements, both within and between unit cells form an interconnecting network, apparently important in maintaining the integrity of the layer. A model which may account for the ICL self-assembly formation* in vivo *and the ICL observed lattice polymorphism is proposed, combining data from the three-dimensional reconstruction work and secondary structure features of the ICL component proteins s36 and s38.*

Keywords: Crystalline eggshell (chorion) layer; *Drosophila grimshawi*; *Drosophila melanogaster* eggshell mutant *fs(1)384*; 3-D reconstruction

Introduction

The *Drosophila* eggshell (vitelline membrane, wax layer and chorion layers) has been studied extensively both as a model system for the study of programmed, differential gene expression during development (Ref. 1 and references therein), and also in order to understand its morphogenesis and structure–function relationship[2,3].

The follicle cells of *Drosophila melanogaster* produce the structural proteins of chorion according to a precise spatial and temporal programme. At the end of oogenesis, the chorion proteins are synthesized by the follicular epithelial cells and secreted onto the surface of the oocyte, where they assemble to form the multilayered chorion. A set of six major (s15, s16, s18, s19, s36 and s38; numbers indicate approximate molecular weights in kDa) and more than 14 minor chorion proteins can be resolved by two-dimensional gel electrophoresis; subsets of these proteins are expressed in a temporally regulated mode during the 5 h of choriogenesis[4–8].

The eggshell consists of several distinct layers: vitelline membrane (VM), wax layer (WL), innermost chorionic layer (ICL), inner endochorion (IE), pillars (P), outer endochorion (OE) and exochorion (EX) (*Figure 1*). These layers perform certain functions, permitting sperm entry-fertilization, exchange of the respiratory gases, mechanical and thermal insulation, water-proofing, resistance to external high pressures, exclusion of microorganisms and hatching. Unique amongst these layers the ICL exhibits a crystalline arrangement of components (Ref. 3 and references therein). Its crystalline structure probably results from postsecretional self-assembly of proteins s36 and s38 during the late stages of choriogenesis, although the details of this process and the precise function of ICL are unknown[9,10]. It has been postulated that the rigidity of ICL helps in the compression of the wax layer onto the vitelline membrane and its porosity allows for the exchange of the respiratory gases[11,12]. Recent work suggests that the eggshell peroxidase which crosslinks chorion proteins at the end of choriogenesis with dityrosine bonds[13] is identical to the s38 protein[14]. Also, s38 has been localized immunocytochemically onto the ICL[14].

The molecular structure of *Drosophila melanogaster* ICL has been studied both by two- and three-dimensional computer reconstruction techniques of isolated and negatively stained specimens (the latter at a resolution of approximately 2.5 nm)[3,15–17]. It was found that the isolated *Drosophila melanogaster* ICL is a single layer, about 12 nm thick and appears to be made up of two types of subunits, each approximately 3 nm in diameter, arranged regularly as groups of four heterodimers in space group C222, with connecting links mainly at the outer surfaces of ICL[3]. The layer exhibits considerable plasticity as might have been expected for a crystalline structure surrounding an oocyte[15–17]

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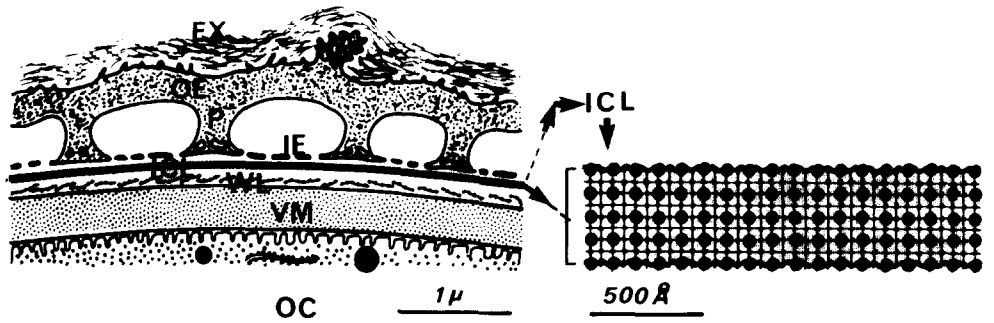

Figure 1 A schematic representation of *Drosophila melanogaster* eggshell showing part of the oocyte (OC) and the eggshell layers, vitelline membrane (VM), wax layer (WL), innermost chorion layer (ICL), inner endochorion (IE), pillars (P), outer endochorion (OE) and exochorion (EX). On the right, the enlargement of the innermost chorion layer (ICL) shows diagrammatically the arrangement of its protein components

In parallel with the structural studies of ICL, a study of the secondary structure of *Drosophila* chorion major proteins has been undertaken, both by theoretical (secondary structure prediction and Fourier analysis of primary structure) and experimental (Fourier transform infrared spectroscopy) methods[10,10a]. A tripartite structure for proteins s36 and s38 was established, which consists of a central domain of several β-sheet strands alternating with β-turns and of two flanking 'arms' containing specific tandemly repeating peptide motifs. These motifs include Tyr at specific locations and might serve for crosslinking. Apparently, they are important structural elements of ICL architecture.

In this report we present the molecular arrangement of the crystalline ICLs from *Drosophila melanogaster* eggshell mutant *fs(1)384*[18] and the Hawaiian species *Drosophila grimshawi*[19], which were calculated from electron microscope images of tilted negatively stained specimens at a resolution of 2.5 nm. Both show structural similarities to the ICL of *Drosophila melanogaster*, but also exhibit distinct differences, most probably related to their function. Also, we discuss a model which may account for the ICL self-assembly formation *in vivo*, combining evidence from the three-dimensional reconstruction work and data obtained from the structural analysis of *Drosophila melanogaster* ICL proteins s36 and s38.

Experimental

Flat sheets of ICL, together with vitelline membrane, were isolated as described previously[3,15], both from *Drosophila melanogaster* eggshell mutant *fs(1)384* and the Hawaiian species *Drosophila grimshawi*, mounted on carbon coated grids and negatively stained with 2% uranyl acetate. Grids were rendered hydrophilic by glow discharging in air for 10 s immediately before use. The glow discharging improved the overall staining of the specimens; no tendency for a 'one-sided' staining was observed. A Philips EM400 electron microscope was used, operating at 100 kV and equipped with a 60° tilt eucentric stage. Tilt series were obtained using the Philips low-dose unit for off specimen focusing and astigmatism correction. The electron dose for each image was approximately 2000 e/nm^2 at the specimen. Micrographs of negatively stained ICL were taken at 6° tilt intervals to a maximum angle of +60°, at a magnification of ×46 000. Focusing and astigmatism correction was carried out off the specimen to avoid beam damage.

The best tilt series were selected by optical diffraction of the micrographs. The areas chosen for densitometry on the EM micrographs were typically squares with a side length of approximately 1 cm. Optical density measurements were performed at 25 μm intervals with an Optronics P-1000 scanning digital microdensitometer. The optical density measurements from image series of tilted specimens were Fourier-transformed and the Fourier coefficients were scaled and refined as described previously[20]. Lattice lines were interpolated by hand in the Z^* (c^*) direction at 40 nm^{-1} intervals from the experimental data and the inverse Fourier transform carried out on a 32 × 32 × 32 grid. Typical values of the unit cell axes were a = 11 nm, b = 11 nm, c = 40 nm (in the case of *Drosophila grimshawi* set initially as a = 11 nm, b = 11 nm, c = 80 nm). The 3-dimensional map was contoured so that the outer boundary corresponded to the maximum rate of change of density. The resolution of the map was 2.5 nm in the plane of the crystal and about 3.5 nm at right angles to the plane. Transmission electron microscopy was performed as described elsewhere[6].

Results

A thin cross-section of a mature *Drosophila grimshawi* follicle, almost perpendicular to the surface of the eggshell (and also to oocyte), showing the crystalline ICL, 70–100 nm thick, is seen in the transmission electron micrograph of *Figure 2(A)*. The corresponding picture taken from a thin section of a follicle of *fs(1)384* is shown in *Figure 2(B)*. It is clearly seen that the ICL of *fs(1)384* is thinner than the ICL of *Drosophila grimshawi*, having a thickness of approximately 17 nm.

Transmission electron micrographs (t.e.m.s.) of isolated flat sheets of ICL, from *Drosophila grimshawi* and *fs(1)384*, negatively stained with 2% uranyl acetate, are presented in *Figures 2(C)* and *2(D)*, respectively, with the electron beam perpendicular to its surface, whereas computer averaged and noise-filtered images from these specimens are shown in *Figures 2(E)* and *2(F)*, correspondingly. These images show well-defined stain excluding regions arranged in groups of four, between well-stained gaps. The unit cell (marked with dotted lines) dimensions for these specimens are: for *Drosophila grimshawi* a = 11 nm, b = 11.3 nm, γ = 89°, and for

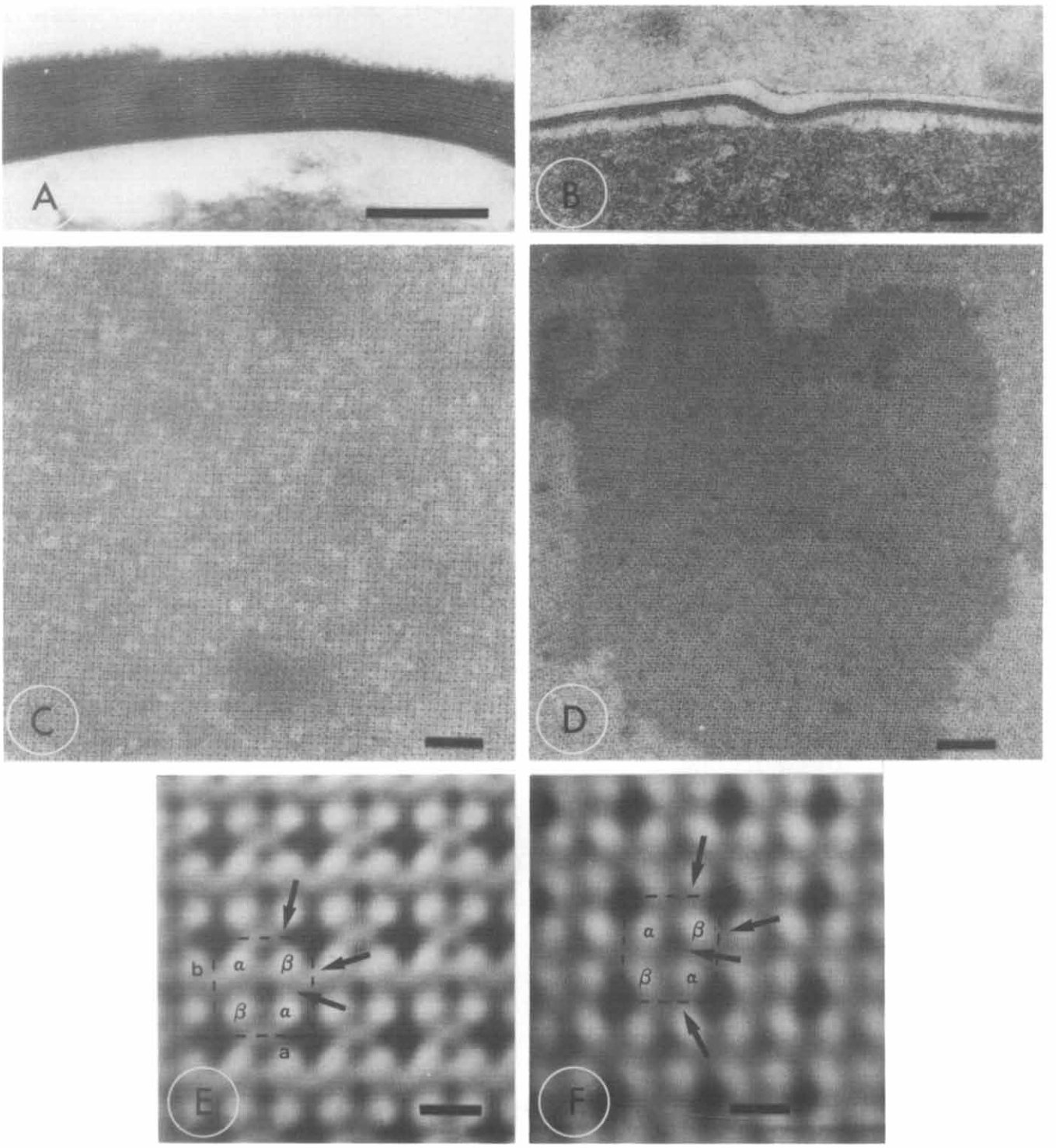

Figure 2 (A) Transmission electron micrograph taken from a thin cross-section, almost perpendicular to the surface of the eggshell (and also to the surface of the oocyte), of a mature *Drosophila grimshawi* follicle, showing the crystalline innermost chorionic layer (ICL), 70–100 nm thick approximately. Scale bar = 140 nm. (B) As in (A), but from a mature follicle of *Drosophila* eggshell mutant *fs(1)384*. ICL thickness = 18 nm. Scale bar = 85 nm. (C) and (D) Whole mount face view of an isolated negatively stained (with uranyl acetate) innermost chorion layer (ICL) from *Drosophila grimshawi* (C) and *Drosophila* eggshell mutant *fs(1)384* (D), respectively, revealing its crystalline arrangement of components. Electron beam almost perpendicular to the ICL surface. Scale bar = 87 nm. (E) and (F) Computer-averaged and noise filtered images of (C) and (D) respectively, showing chosen unit cells. The asymmetric unit, in each case, consists of two non-identical stain excluding regions, marked α and β, whereas pairs of asymmetric units are related by a twofold axis perpendicular to the plane of ICL. For further details see text. Scale bar = 6.9 nm

fs(1)384 $a = 11.4$ nm, $b = 11.7$ nm, $\gamma = 87°$. However, these dimensions vary from specimen to specimen, being $a = 10.5 \pm 1.5$ nm, $b = 10.5 \pm 1.5$ nm, $\gamma = 90 \pm 15°$. Similar variations have been observed in the ICL of *Drosophila melanogaster* and they are probably related to its plasticity[3,15,16]. Each unit cell appears to contain two asymmetric units related by a two-fold axis, in projection. Each asymmetric unit consists of two non-identical stain-excluding structural units, marked α and β and the plane group symmetry appears to be, at least p2.

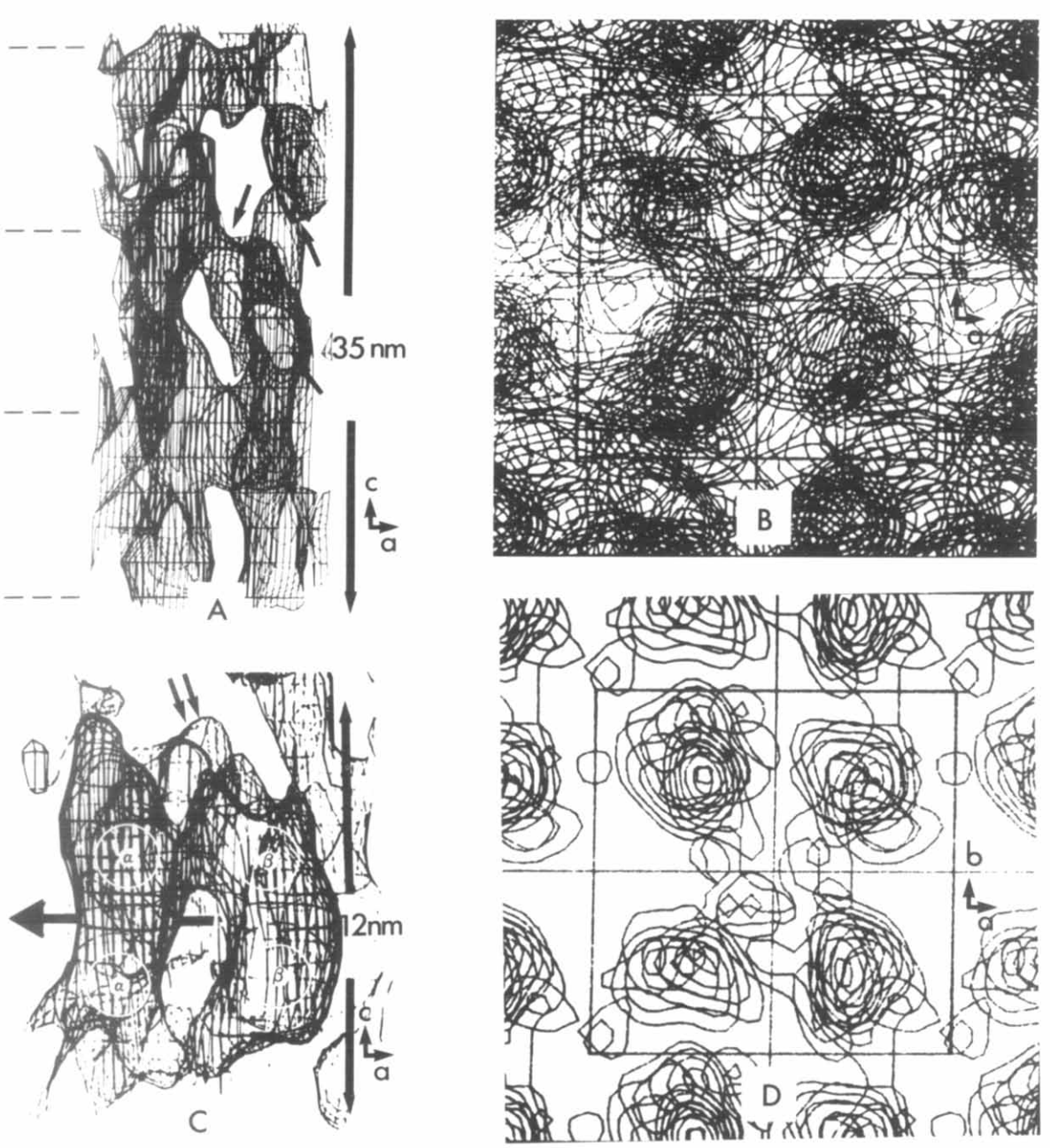

Figure 3 (A) and (C) Views of the three-dimensional density maps of *Drosophila grimshawi* (A) and *Drosophila* eggshell mutant *fs(1)384* (C) respectively, on an interactive graphics screen, along a direction inclined at an angle of approximately 20° to the plane of ICL. The map is contoured so that the outer boundary corresponds to the maximum rate of density and contours are drawn at arbitrary intervals. Contoured areas represent stain excluding regions. The *a*-axis is parallel to the plane of ICL and the *c*-axis is perpendicular to ICL. For further details see text. (B) and (D) Projections down the crystallographic *c*-direction perpendicular to the plane of the calculated 3-D maps of *Drosophila grimshawi* (B) and *Drosophila* eggshell mutant *fs(1)384* (D). Contouring as in (A) and (C) above. For details see text

As in the case of *Drosophila melanogaster*, the symmetry in three dimensions was not prejudged and all subsequent analysis was carried out by assuming the space group symmetry as p1. Weak linking density (*Figures 2(E)* and *2(F)*, arrows) appears to crosslink structural elements in each unit cell and between unit cells, forming an interconnecting network, which seems to be important in maintaining the integrity of the layer.

Figure 3 shows the three-dimensional density maps from *Drosophila grimshawi* and *fs(1)384*. These were calculated from two combined sets of data in each case, which were selected on the basis of the similarity of their zero tilt computer-filtered images. *Figures 3(A)* and *3(C)* are views on an interactive graphics screen, of the density maps for *Drosophila grimshawi* and *fs(1)384* respectively, along a direction at an angle of approximately 20° to the plane of the crystalline layer, whereas *Figures 3(B)* and *3(D)* are projections along an axis perpendicular to the layer. From the views almost parallel to the plane of the crystalline ICL and the projections perpendicular to the layer, it can be seen that, in both cases, stain channels (non-contoured areas), approximately 2.5–3.5 nm in diameter, penetrate through the layer.

The *Drosophila grimshawi* map (*Figure 3(A)*), shows that the crystal thickness is about 35–40 nm and the ICL consists of three sheets (dotted lines) of structural units

(it has a thickness of three unit cells), whereas the *fs(1)384* map suggests that the isolated ICL consists of a single sheet of structural units, approximately 11–13 nm in thickness, a phenomenon also observed in *Drosophila melanogaster*[3]. In both cases, each unit cell consists of tetramers of stain excluding material, elongated along a direction perpendicular to the layer, roughly 3–4 nm in diameter and 10–11 nm in length. Each elongated structural unit appears to be divided into two, roughly equal, domains (circles) related with a dyad axis in the plane of the crystal (*Figure 3(C)*, arrow). In the *Drosophila grimshawi* map (*Figure 3(A)*), successive structural units are interconnected with dense material, in directions perpendicular and parallel to the layer, both, within and between unit cells. In the *fs(1)384* map (*Figure 3(C)*), the elongated (most probably dimeric) structural units are cross-linked with stain-excluding material at the edges of the crystalline layer (double arrows).

Discussion

The results of these reconstructions suggest that the innermost chorionic layer, as we isolate it, has a thickness of one unit cell (12–13 nm) in *fs(1)384* and a thickness of three unit cells (35–40 nm) in *Drosophila grimshawi*. They are in agreement with measurements made on platinum shadowed ICLs from both species in comparison with TMV as thickness standard, from observations made after thin sectioning of grids with negatively stained ICLs and from s.t.e.m. measurements of electron scattering of unstained isolated ICLs (data not shown). This is a puzzling observation if compared with the results seen in thin sections of the intact eggshells which show evidence for a multilayered structure, having a thickness greater than that of the isolated ICLs in both species. The same phenomenon, however, has been observed in *Drosophila melanogaster*[3]. A plausible, though not necessarily convincing, explanation might be that the multilayered structure dissociates into sublayers during the ICL isolation procedure: it has always been observed that the ICL expands laterally when reaching an air–water interface.

From the diffraction patterns (optical and computer calculated) of the images of negatively stained isolated ICLs (data not shown), the resolution of the density maps is estimated to be 2–2.5 nm in the plane of ICL and limited to about 3.5 nm at right angles to the plane (resolution along this direction is lower because of the missing cone of data at tilt angles greater than 60°). The loss of resolution along a direction perpendicular to the layer obscures the clear separation of each elongated structural unit into two domains, which appear to be related by a twofold axis in the plane of the crystal. This is obvious after a careful examination of the maps and suggests that each unit cell contains eight subunits forming four dimer pairs, two of one type, α, and two of another type, β (*Figure 3(C)*) and is reminiscent of the model proposed for the ICL of *Drosophila melanogaster*[3]. Obviously, however, there are important differences from the model of *Drosophila melanogaster*. In *fs(1)384*, one type of subunit (type β) appears to be clearly smaller than the corresponding subunits of *Drosophila melanogaster*. Nevertheless, the isolated ICL has a thickness of only one unit cell, as in *Drosophila melanogaster*. In *Drosophila grimshawi*, the subunits have similar size with those of *Drosophila melanogaster*, whereas the ICL has a thickness of three unit cells. Cross-linking density between successive unit cells along a direction perpendicular to the layer evidently contributes to the rigidity and integrity of the layer. These obvious differences most probably reflect variations in the amino acid sequences of the proteins forming ICL in the three different species. Unfortunately, the amino acid sequences of proteins s36 and s38, the main constituents of ICL[14] have not been reported so far for *Drosophila* eggshell mutant *fs(1)384* and the Hawaiian species *Drosophila grimshawi*. In a recent study, the sequences of all the other major proteins which constitute the eggshell of *Drosophila grimshawi*, s15, s16, s18 and s19, have been determined, but not of s36 and s38[21,22]. The published sequences of the low molecular weight *grimshawi* eggshell proteins show extensive, conservative homologies with those of *Drosophila melanogaster*, but also exhibit distinct localized variations. From the reconstruction data it might be expected that the basic sequence features should be conserved, considering the profound similarity of the gross structural features of ICL in both species. However, we predict also variations, which should account for the crossbridges between successive unit cells in a direction perpendicular to the layer.

Following the arguments, described in detail by Margaritis *et al.*[3], it is expected that the molecular weight of each structural subunit is approximately 35 000 Da, in agreement with observations which demonstrate s36 and s38 as constituents of ICL[14]. The value of 75 000 Da per monomer, reported by Akey *et al.*[16,17] based on the stain-excluding volume present in their map, appears not to be consistent with our observations.

Recent analysis of the amino acid sequences of proteins s36 and s38[10], implies a tripartite structure for each protein: a central domain which consists of several β-sheet stands alternating with characteristic β-turns or loops, forming, most probably, an antiparallel β-sheet structure, and two flanking 'arms' containing characteristic tandemly repeating peptide motifs. Both 'faces' of the β-sheet have a profound hydrophobic character, whereas the 'arms' contain characteristic tandem repeats of Tyr and long stretches of alanines predicted as α-helices. The β-sheets of the central domain might fold into a β-barrel type of structure or simply form a twisted β-pleated sheet, generating a globular 'core' for both proteins s36 and s38 with a diameter of the order of 3–4 nm. This may correspond to the globular features of subunits and of the reconstruction maps. The 'arms' of the proteins, with tandemly repeating peptides containing Tyr at specific locations, are ideal candidates for the formation of interconnecting bridges between the globular subunits by covalent crosslinks, di-tyrosine and tri-tyrosine bonds. These are known to harden and make insoluble the eggshell during the late choriogenetic stages through the action of a peroxidase *in vivo*[23]. The rod shape of the intermolecular crosslinks and their cross-sections of the order of 1–2 nm, are in good agreement with secondary structure predictions for these parts of the molecules which suggest an α-helical type of structure[10]. Furthermore, the well documented inherent conformational flexibility of protein α-helices[24], allows us to postulate that these regions of the molecules are primarily responsible for the observed lattice polymorphism[15,16] and the intrinsic ability of ICL to be a curved crystalline layer surrounding an oocyte.

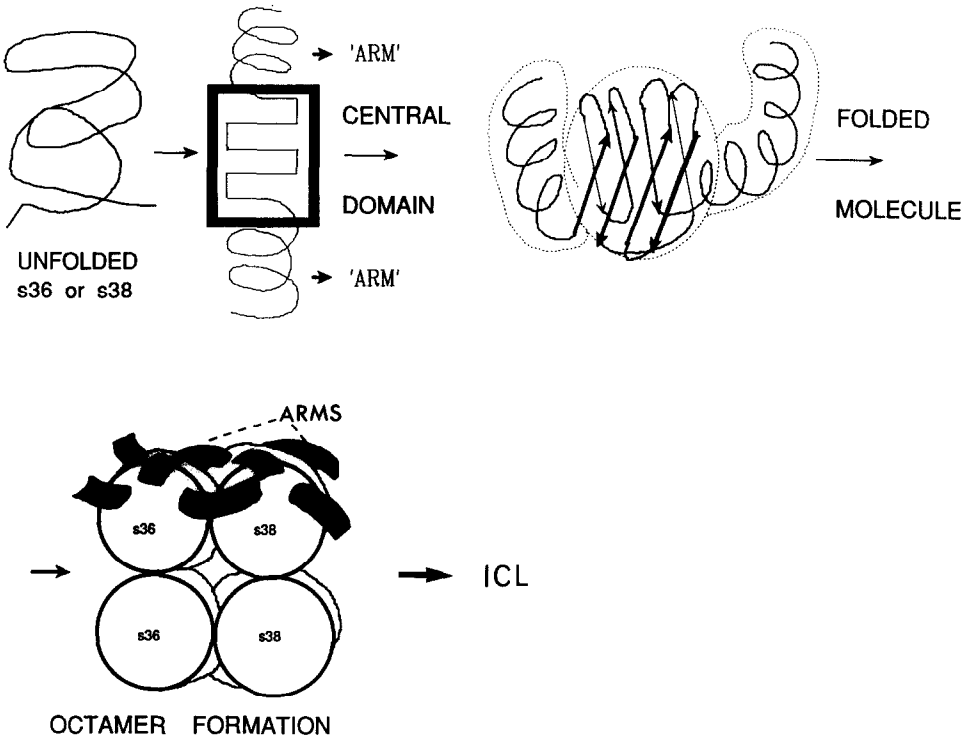

Figure 4 A schematic model of a proposed self-assembly mechanism of ICL formation *in vivo*. Secreted unfolded eggshell proteins s36 and s38 fold into tripartite structures having a globular β-sheet core (see text) and two 'arms', mostly α-helical, rich in tyrosines, which serve for crosslinking. Assembly of folded s36 and s38 molecules into octamers and ICL formation proceeds via intermolecular crosslinking with di-tyrosine bonds. In the octamer, only the hypothetical crosslinks in a tetramer are shown

From the above evidence, the schematical model shown in *Figure 4*, might represent the self-assembly mechanism of ICl formation *in vivo*.

The precise functional role of ICL is as yet unknown, although from the reconstruction and biochemical data several possible roles have been postulated[2]. Its evolutionary conservation, in at least six orders of insects[25], implies exceptional properties not shared by any other eggshell layer. However, there are indications to suggest that its main role is to act as a barrier to water permeability in conjunction with its underlying, nearest to the oocyte, wax layer. The oocyte is waterproofed by the presence of a highly hydrophobic, very thin, wax layer which is held in contact with the vitelline membrane, in a sandwich-like arrangement between the vitelline membrane and the ICL, by the pressure exerted on to it from the overlying ICL (*Figure 1*; see also Ref. 2). Wax molecules also penetrate within the stained filled 'channels' of the ICL seen in the 3-D reconstruction maps[25a], creating a hydrophobic environment but, apparently, are removed during the ICL isolation procedure. Our unpublished permeability experiments, done *in vitro*, on isolated VMOs (vitelline membrane oocytes[6]), involving the use of several solvents, strongly support the waterproofing properties of the ICL in conjunction with the wax layer. In this respect, it is important to note that the eggshell mutant *fs(1)384* exhibits permeability problems. The mutation has been mapped to be on the X-chromosome[18] and the genes coding for s36 and s38 are located on the same chromosome[1]. One of the two subunits of ICL appears to be smaller in *fs(1)384* than in *D. melanogaster* in the reconstruction maps (see Results) and this alteration may be correlated with the permeability problems. Alternatively, the defective endochorion[25a] might be responsible for the non water-proofness of the *fs(1)384* oocyte. Further work on this mutant should identify whether alterations in the amino acid sequences of either s36 or s38 are actually responsible for the abnormal permeability properties of this eggshell. In a similar context, it is interesting to observe the difference in ICL thickness between *Drosophila melanogaster* and the Hawaiian species *Drosophila grimshawi*[19], seen in cross-sections, also evident in the isolated layers. Apparently, these might be related to the micro-environment of the egg-laying substrate of the two species: *D. melanogaster* oviposits in a variety of substrates, under a variety of conditions (humid and dry), whereas *D. grimshawi*, evolved under unique geological circumstances, oviposits in the bark, under an environment which is mostly humid, containing decaying substances[19]. It might be rewarding to record at molecular level the amino acid alterations in the sequences of s36 and s38, which cause the formation of a multilayered ICL structure in *D. grimshawi*, presumably, more impermeable to water in conjunction with the wax layer.

Recent work[26] on the eggshell protein sequences of the med fly *Ceratitis capitata* (Diptera/Tephritidae), an insect of great economic importance, having an evolutionary distance of approximately 120×10^6 years from *Drosophila melanogaster*[27,28], which contains an ICL similar in structure in its eggshell[29], suggests a similar fold of the highly homologous in sequence, central domain of its proteins to those of *Drosophila melanogaster*; the protein 'arms', apparently, exhibit different folding patterns[26]. The unique structure of the central domain, which, most probably forms the globular part of the ICL subunits and its highly conserved character throughout evolution, indicates that this domain is well tailored to play its functional role and further suggests its universal appearance in other insects known to employ an innermost chorionic

layer in their eggshell architecture[2]. More refined biochemical and structural work is needed to prove the validity of our model and accurately identify the structural component parts of ICL.

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