Growth of protein-doped rhombohedra in the calcitic shell of craniid brachiopods

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The secondary shell of the living inarticulated brachiopod *Neocrania* consists of calcitic laminae interleaved with organic sheets, predominantly a 44 kDa protein with high levels of aspartic acid–asparagine and glutamic acid–glutamine. Laminae consist of tabular (01.4) rhombohedra that are composed of spherular or rhombohedral granules, *ca.* 30 nm in size. Rhombohedra increase by planar or spiral growth as granular, monolayered plates that commonly act as foundations for multilayered tablets up to 300 nm or so high. Rough (0k.l) faces, kinked by cleavage, usually develop at either end of the long diagonals of rhombohedra, the edges of which may support ramparts that can accrete centripetally to enclose any organic material situated at tablet centres. Induced degradation by proteinase K shows that sectors subtended by (0k.l) steps are doped by a fibrous polymer, identified as an exclusively intralaminar glycosylated 60 kDa protein. A 44 kDa protein has also been extracted from laminae and is presumably incorporated into tablets by centripetally growing ramparts.

Keywords: protein-doped calcite; spirally growing rhombohedra; organocalcitic brachiopod shells

1. INTRODUCTION

The structure of the secondary layer of the calcitic shell of the living brachiopod *Neocrania* has been known since 1970 (Schumann 1970; Williams & Wright 1970). The layer consists of spirally growing tablets that coalesce into sheets (laminae) interleaved with membranes (Williams 1970). Two recent studies have prompted a review of this interpretation of laminar growth. Weedon & Taylor (1995) described how bleach etched hillocks within crystallographically constrained sectors of tablets in the laminar shell of cyclostome bryozoans. Brown (1998) reported the extraction of intracrystalline proteins with calcium-binding properties from the craniid shell and the etching of preferred sectors of laminar rhombohedra by bleach. Our investigations show that these manifestations of induced laminar degradation are linked because etches represent imprints of proteins adsorbed along rough faces of rhombohedra while other macromolecules are incorporated by centripetal growth of tablets.

2. MATERIAL, METHODS AND TERMINOLOGY

The materials used were manually cleaned shells of *Neocrania anomala* (Müller) from the Firth of Lorne, Scotland. Fractured pieces were viewed under Cambridge 360 and Hitachi 900 scanning electron microscopes (SEMs) in the dried state, after being immersed in distilled water, treated with sodium hypochlorite (bleach), digested in proteinase K or subjected to plasma etching. Polished sections of shells mounted in London resin were studied by microprobe and energy dispersive X-ray (EDX) facilities. Intercrystalline and intracrystalline organic residues

were extracted and analysed under clean room conditions. The techniques and facilities used are described in Williams *et al.* (1998).

The crystal form of calcite in the secondary shell of *Neocrania* is a tabular rhombohedron, (10.4), lying in the plane of laminar succession. Faces sharing edges with the (10.4) face, which may exceed 5 µm diagonally, are seldom more than 300 nm high and will be referred to as 'steps' (figure 1a). Their indices are also used to distinguish edges shared with the (10.4) face. Up to four other minor faces (steps) are variably developed with edges parallel with the diagonals of the (10.4) face and they and their edges are identified by general indices (figure 1a). The terms used to describe the chemico-structure of rhombohedra are given in figure 1b.

3. RESULTS

(a) Interlaminar substrates

The organic sheets interleaved with calcitic laminae consist of an electron-dense mesh containing close-packed, electron-light vesicles *ca.* 20 nm in diameter (figure 2b). The sheets, usually spaced at 30–300 nm, may terminate abruptly or branch at acute angles or in near-vertical steps (figure 2a). Those immediately succeeding stepped zones are commonly fragmented. Sporadically occurring blocks of the same composition and up to several hundred nanometres wide connect contiguous sheets (figure 2a).

Under the SEM, a tension-cracked, organic cover normally obscures mineralized surfaces of dried shells (figure 2e), although immersion in water may cause some degradation, presumably by autolysis and bacterial activity (figure 4f). Such covers are differentially digested by proteinase K leaving residual patches with

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Figure 1. Crystallographic and chemico-structural terminology. (a) Indices used to define the faces (or steps) of tabular rhombohedra, confirmed by X-ray diffraction as (10.4), that form the laminae of the secondary shell of *N. anomala*; long and short diagonals are shown as dotted lines and rough faces—steps as cleavage-kinked. (b) Stylized etched tablet (tb) on plate (pl) with banded (gb), centrally depressed (cd), internal surface composed of granular mosaic (mo), coated with proteinaceous infill of 44 kDa (if) partly covered by ramparts (ra) accreting on a foundation of banded rhombohedra (rb); the etched (0k.1) sector (es) with ridges and hillocks delineates sites doped by 60 kDa protein along kinked steps (ks).

open, anastomosing and dendritic trails a few nanometres wide. They are more quickly and comprehensively degraded by bleach and plasma etching which transiently expose two other organic constituents. In low concentrations of bleach, an open network of fibres *ca.* 15 nm thick is rarely preserved in etch pits and along sutures in mineralized substrates (figure 2c). Plasma etching has exposed another network of coarser, radiating strands up to 150 nm thick with thinner cross-bars (figure 2d). This network occurs in oval patches up to 10 μ m long covering surface tablets and their substrates.

The commonest interlaminar polymer extracted is a calcium-binding 44 kDa protein with high levels of aspartic acid–asparagine, glutamic acid–glutamine and serine (Brown 1998). The protein also occurs within laminae.

(b) *Laminae of secondary layer*

Laminae are organocalcitic sheets, seldom more than 300 nm thick but normally many square micrometres in area. They are essentially disposed horizontally, bending outwards as walls of densely distributed canals (punctae) that contain caecal outgrowths of the mantle (figure 3a). A lamina is usually made up of many flat-lying rhombohedra, separated from one another by sutures that sporadically enclose polygonal ‘windows’ to reveal inliers of older, outer laminae (figure 4i). Epitaxial alignment between and within laminae is localized but can extend through at least ten laminae. Rhombohedra with (10.4) faces are composed of spherular or rhombohedral aggregates of granules ranging diametrically–diagonally from 15 to 50 nm in size (figure 3c,f). They enlarge by spiral (figure 3e) or planar (figure 3f) growth on interlaminar substrates and are found at all stages of accretion, virtually from nucleation, on the internal surface. Despite their chemico-crystallographic homogeneity, rhombohedra are morphologically distinguishable as monolayered plates and multilayered tablets with different patterns of growth and macromolecular adsorption.

(i) *Plates*

Plates occur discretely or, more commonly, as foundations for tablet growth. The smallest plates found were between 150 and 500 nm in the long diagonal (figure 3b,c), with (0k.1) steps, roughened by rhombohedral kinks, generally developing in larger plates (figure 3d). At all sizes, plates are essentially flexible monolayers of spherular granules arranged in mosaics or zoned in growth bands *ca.* 30 nm wide. Their surfaces are either flat or centrally depressed where granules are more dispersed. Such depressions normally slope towards one of the (10.4) edges which may be breached (figure 3b,c). They may also extend as grooves more or less aligned with the long diagonal. We assume that depressed plates are spiral growths initiated at the edges of tilted rhombohedra protruding above interlaminar substrates (figure 3d).

(ii) *Tablets and ramparts*

Tablets consist of up to eight layers of granular mosaics that, like plates, accrete by planar or, more commonly, spiral growth (figure 3e,i). Degraded surfaces are variably zoned parallel to the edges of tablet faces, around flat granular or depressed proteinaceous centres (figure 3e).

Rough (0k.1) steps are usually well developed in larger tablets and the triangular sectors of growth they subtend with the rhombohedral centres may be sharply delineated (figure 4e). Rough (h0.1) steps, when developed, are short and remain so during growth, as is shown by their generation of banding in parallel strips (figure 4d).

Just within the surface edge of a tablet, mosaics of granules may give way to bands of rhombohedra *ca.* 40 nm long (figure 3j). The bands are the foundations of commonly occurring, presumably rapidly secreted walls (ramparts), up to 600 nm high (figure 3i). Ramparts can develop on tablets or plates less than 1 mm long diagonally. They are, however, much commoner along the (10.4) edges of spirally growing, larger tablets (figure 3i), including those twinned on (h0.1) faces (figure 4j). Ramparts are composed of rhombohedral aggregates of granules in

Figure 2. Integument of *N. anomala*. (a, b) TEM sections of decalcified integument, stained with aqueous uranyl acetate and aqueous lead citrate, showing electron-dense meshes with electron-light vesicles (ve) as sheets (sh) originating from microvilli (mi) of apical plasmalemma and disrupted above a tablet (tb) and as infills (in) between laminae in (a). (c–e) SEMs of internal surfaces. (c) Treated with bleach (10% by volume) for 1 h with etch pits (ep) in a tablet (tb) and revealing a fibrous network (fn) in the substrate. (d) Plasma etched for 4 h showing network of strands over a tablet. (e) Immersed in distilled water for 72 h, tension cracked (tc) interlaminar sheet overlying a tilted plate (pl).

bands parallel to the (10.4) edges of tablets (figure 3k). They accrete centripetally as well as vertically and may coalesce to cover organic infills of the central depression (figure 3k).

(iii) Etched sectors

Differential etching of the surfaces and steps of plates and tablets exposes the fine relief of the calcite and excavates sites of organic concentrations. The effects are generally more evident on underlying laminae exposed by degradation of organic substrates than on biomineralized structures being secreted at death (figure 4i). This suggests that, in addition to some protein doping in the later stages of laminar formation, many organocalcitic microstructures are highly degradable *in statu nascendi* and can even be destroyed by autolysis.

The simplest etched features are slits or narrow clefts, extending between the centre and the acute-angled corners of rhombohedral plates, parallel to the long diagonal (figure 4a,b). Growth bands are not dislocated by the kinked sides of clefts, which end centrally in a granular mosaic, less than 500 nm in diameter (figure 4c). Opposite clefts in flat plates are aligned with each other and arise from a common centre, but those in spirally growing plates are sinistrally and dextrally displaced as are their separate centres (the ‘emergency points’ of screw dislocation) in clockwise and counterclockwise spirals, respectively (figure 4b,c). These shifts are presumably a function of rhombohedral distortion by spiral growth.

The removal of narrow strips of plates along their long diagonals by proteinase K confirms that granules within those zones are invested in a protein that had been adsorbed at the junctions of the (01.4) $\wedge$ (11.4) and (10.4) $\wedge$ (01.4) steps, virtually from plate inception. Such

adsorption is rare along the narrow, ladder-like strips (or linear traces) of the step junctions enclosing the short diagonal (figure 4d).

Surface etching of tablets by induced degradation is restricted to triangular zones (figure 4a,e) with bases at or just within the (0k.1) steps and apices, subtending angles up to 70°, at central depressions (‘0k.1 sectors’). The sides of excavated (0k.1) sectors are more or less parallel to (10.4) edges but are commonly unequally developed with one side forming a step and the other a break in slope; both are kinked by rhombohedral cleavage. Degraded sites vary from hillocks to labyrinthine walls or parallel ridges (figure 4h). Deeply etched residues usually rest on basal plates and are aligned with rhombohedral cleavage. Other etched sectors consist of gently inclined, granular sheets, *ca.* 20 nm thick (figure 4f). These sheets are not traceable in flanking, wholly mineralized sectors and owe their distinctiveness to proteinaceous interleaves. (0k.1) sectors are identifiable in sections treated with bleach or enzymes as edges roughened by differential etching along the cleavage and pits (figure 3g,h).

Ramparts can grow on the surfaces of tablets with etched (0k.1) sectors. Indeed, pit-free borders that occasionally intervene between (0k.1) edges and bases of etched sectors would be suitable sites for the nucleation of ramparts. The subsequent centripetal growth of ramparts can result in ledges coalescing inwardly to enclose organic infills in the central depressions of tablets (figures 3k and 4g). Such ‘intralaminar’ deposits are really part of the organic interlaminar component of the secondary shell.

Degradation of laminae underlying the internal surface of the shell exposes plates and tablets coalesced within mosaics or concentric bands of granules with sutures

Figure 3. SEM views of internal surfaces and fracture sections of secondary shell of dorsal valves of *N. anomala*. (a, c, e, k) Digested by proteinase K in phosphate buffer. (b, g, h) and (f, i) Treated with 10% and 20% bleach by volume for 6 h, respectively. (d, j) Treated with phosphate and hepes buffers, respectively. (a) Fracture section showing disposition of laminae (la) relative to internal surface (in) and punctae (pu). (b, c) Immature and newly formed, granular rhombohedral plates with central depressions (cd). (d) Growth-banded plate with sparse etch pits along long diagonal between rough steps (0k.l) (rs) on interlaminar substrate (is) raised by tilted tablet (tr). (e) Clockwise spiral tablet system with centripetally growing rampart (ra) and (0k.l) and (h0.l) rough steps (rs) and (rl), respectively. (f) Planar tablet on interlaminar substrate (is). (g, h) Fracture sections of (0k.l) sectors (es) of tablets, etched along cleavage and pits (ep), in relation to smooth, growth-banded (1 l.4) sector (gb), with a window (wi) at suture junction. (i) Spiral tablet system with centripetally growing ramparts (ra) elevated above interlaminar substrate. (j) Corner of tablet with growth banding (gb), bands of larger rhombohedra (rb) parallel with (01.4) step and granular mosaic (mo) within rough (0k.l) step. (k) Tablets on plates (pl) with growth-banded (gb) ramparts (ra) covering the central depression.

Figure 4. SEM views of internal surfaces of secondary shell of dorsal valves of *N. anomala*. (a, b, c, g, i) Digested by proteinase K buffered by phosphate for (a) and hepes. (d, j) Treated with hepes and phosphate buffers, respectively. (e, h) Treated with bleach at 10% and 20% by volume for 6 h, respectively. (f) Degraded by autolysis in distilled water. (a, b) Slits (sl), clefts (cl) and etched (0k.l) sectors (es) in spirally growing plates and tablets. (c) Two dextrally shifted central depressions (cd) of clefts excavated in counterclockwise, spirally growing rhombohedra. (d) Ladder-like growth banding (gb) of rough (h0.l) step flanked by sectors with granular mosaics (mo). (e, f) Etched sectors (es) within or delineating rough (0k.l) edges (rs), bounded by etched, kinked steps (eb) parallel with (1 1.4) and (01.4) edges and containing hillocks (hi) and granular sheets (gs), respectively. (g) Tablets, twinned on (0k.l) plane (?) with infill centres (if) revealed by degradation of centripetally grown, granular (gr) cover of banded (gb) ramparts (ra). (h) Laminar etching along sutures (su) with rhombohedral labyrinths (lb), ridges and hillocks (hi). (i) Rhombohedral grilles (rg) in laminar substrates. (j) Spirally growing rhombohedral sets twinned on (h0.l) faces emerging from interlaminar substrate (is).

sporadically enlarged by rhombohedral etch pits (figure 4*h*). Etched (0*k.l*) sectors in various stages of development and degradation occur together with rare rhombohedral and subtriangular faces, up to 4 µm or more long, that have been differentially etched into rhombohedral grilles or slats, parallel to a {10.4} edge (figure 4*i*). We assume that these structures result from adsorption of abnormal quantities of protein along the cleavage.

4. CONCLUSIONS

Induced degradation of laminae in craniid shells, including enzymic digestion, showed that excavated sites contained organic, predominantly proteinaceous residues. The efficacy of enzymic digestion in revealing the nanometric relief of laminae suggests that their constituent granules are enclosed in a protein, which is presumably water soluble as traces of it are not preserved in decalcified sections prepared for the transmission electron microscope (TEM). Protein also occurs as infills along sutures and within rhombohedral tablets. TEM studies show that the infills, like those occupying windows (figure 2*a*), as well as particles in some tablets, are electron-dense proteins. The protein is assumed to be the electron-dense mesh of interlaminar organic sheets, which is associated with vesicles also secreted by microvilli. It is tentatively identified as the 44 kDa protein extracted from both laminae and interleaves.

Tablets are doped with proteins in at least two ways. Centripetal growth of the top granular layers of ramparts can trap organic residues coating medial depressions of tablets (compare with fig. 4 in Towe (1980)). This process is assumed to be mainly responsible for incorporating the 44 kDa protein within tablets. The other sites of doping are (0*k.l*) sectors. They vary from slits in plates, representing cleavage kinks along the long rhombohedral diagonal, to wide sectors in tablets based on strongly developed, kinked (0*k.l*) steps. Along slits, the protein is secreted as kinked strands at the lengthening junction between the growing (1 $\bar{1}$.4) and (0 $\bar{1}$.4) steps. In sectors, it forms strands aligned with {10.4} cleavage, with offsets partly or completely enclosing granules so that degradation exposes calcitic ridges and hillocks and releases detached granular aggregates. Secretion of the protein can be periodic with repeated, sustained doping of the kinks in the (0*k.l*) steps to form more or less continuous organic bands, many nanometres thick, parallel with the sector base. Secretion can also be terminated within the base of a sector but such protein-free bands appear to be composed of granular rhombohedra which may become sites for rampart growth. The protein also forms sheets interleaved with granular monolayers; both constituents are restricted to (0*k.l*) sectors. The sectoral restriction in the secretion of this fibrous protein suggests that it is the exclusively intralaminar, glycosylated 60 kDa protein (Brown 1998). Sporadically preserved strands in etched sectors and sutures (figure 2*c,d*) may be remnants of this proteinaceous network.

Why (0*k.l*) sectors are susceptible to protein doping has yet to be answered. In reviewing sectoral zoning in carbonates, Reeder (1991) ascribed the partitioning of trace element adsorption to the differential growth of steps on non-equivalent faces. Fernandez-Diaz *et al.*

(1996) experimentally confirmed that Mg doping inhibits CaCO₃ nucleation and promotes kinking and crystal deformation. In our craniid studies, no displacement of banding by slits or etched sectors was seen and the morphological variation of tablets and plates was orthodox. The Mg content (up to 8.6%) of the shell of *Neocrania* is high compared with less than 1.5% in the calcitic shells of other brachiopods (Jope 1965). However, neither sectoral nor concentric zoning involving Mg could be determined by electronic superprobe analysis of untreated or bleached (5% by volume for 6 h) rhombohedra (H. Höfer and T. Stachel, personal communication).

Sectoral zoning, revealed by differential etching of carbonate shells, is found in other phyla. That found by M. J. Weedon in etched laminae of the bryozoans *Biflustra* and *Heteropora* is like that of *Neocrania* and doping by a homologous protein is a possibility, particularly as bryozoan laminae also interleave with vesicular proteinaceous sheets (Tavener-Smith & Williams 1972, fig. 97).

Sectorally zoned tablets are found in molluscan shells which, however, are chemico-structurally different from those of lophophorates. Molluscan interlaminae consist of calcium-binding, aspartic-acid-rich macromolecules coating a layer of β-chitin between sheets of fibroin-like protein (Weiner & Traub 1984). Moreover, the carbonate laminae are polymorphous (Falini *et al.* 1996) and sectoral doping is best known in the aragonitic tablets of bivalves (Mutvei 1977). Even these orthorhombic tablets are differently structured as doped sectors consist of lamellar successions of aragonitic granules and organic sheets.

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