On the Formation and Functions of High and Very High Magnesium Calcites in the Continuously Growing Teeth of the Echinoderm *Lytechinus variegatus*: Development of Crystallinity and Protein Involvement

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Key Words

Echinoderm tooth structure • Magnesium calcite, high, very high • Syncytial proteins

Abstract

Sea urchin teeth grow continuously and develop a complex mineralized structure consisting of spatially separate but crystallographically aligned first stage calcitic elements of high Mg content (5–15 mol% mineral). These become cemented together by epitaxially oriented second stage very high Mg calcite (30–40 mol% mineral). In the tooth plumula, ingressing preodontoblasts create layered cellular syncytia. Mineral deposits develop within membrane-bound compartments between cellular syncytial layers. We seek to understand how this complex tooth architecture is developed, how individual crystalline calcitic elements become crystallographically aligned, and how their Mg composition is regulated. Synchrotron microbeam X-ray scattering was performed on live, freshly dissected teeth. We observed that the initial diffracting crystals lie within independent syncytial spaces in the plumula. These diffraction patterns match those of mature tooth calcite. Thus, the spatially separate crystallites grow with the same crystallographic orientation seen in the mature tooth. Mineral-related proteins from regions with differing Mg contents were isolated, sequenced, and characterized. A tooth cDNA library was constructed,

and selected matrix-related proteins were cloned. Antibodies were prepared and used for immunolocalization. Matrix-related proteins are acidic, phosphorylated, and associated with the syncytial membranes. Time-of-flight secondary ion mass spectroscopy of various crystal elements shows unique amino acid, Mg, and Ca ion distributions. High and very high Mg calcites differ in Asp content. Matrix-related proteins are phosphorylated. Very high Mg calcite is associated with Asp-rich protein, and it is restricted to the second stage mineral. Thus, the composition at each part of the tooth is related to architecture and function.

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Introduction

The sea urchin tooth is a complex, compartmented structure which grows continuously from the aboral plumula region (functionally the equivalent of the root por-

Abbreviations used in this paper

Lv	<i>Lytechinus variegatus</i>
PO	preodontoblasts
SEM	scanning electron microscopy
WAXS	wide angle X-ray scattering

tion of the developing vertebrate rat incisor) to a fully mineralized adoral, self-sharpening incisal tip. Growth begins at the sheath-like bulbous structure of the plumula, an epithelial sac exposed to the coelomic fluid. A layer of undifferentiated mesenchymal cells, i.e. preodontoblasts (PO), coats the basement membrane along the internal aspect of the epithelium. Morphogenesis begins as the PO mature into odontoblasts and these quickly migrate and move into the enclosed space. The migration is not general, but finger-like cytosolic projections guide the cells into sheets in which the cell membranes fuse and form a multinucleated syncytium in each layer [Märkel et al., 1986]. Membrane-bound spaces or vacuoles within the cellular syncytium become the sites for primary mineral nucleation [Märkel et al., 1969, 1986; Kniprath, 1974]. Ultimately, intrasyncytial first stage mineralization leads to the formation of first stage (high Mg, i.e. 5–15 mol% Mg) calcite plates, needles, and prisms. As the tooth grows in the adoral direction and takes on its mature form, the cells in the syncytia remain alive but occupy an ever smaller fraction of the tooth's volume. The first stage mineralized elements become very complex in organization and ultimately become fused together, cemented by second stage calcite of quite a different composition (very high Mg, i.e. 30–40 mol% Mg). There is strong evidence that the proteins associated with very high Mg secondary stage calcite are different from those related to primary calcite [Alvares et al., 2007]. Despite many differences in the shape, orientation, and composition of the structural elements of the tooth, it diffracts as if it were a single crystal: all of the plates, primary and secondary, and needles and prisms have essentially identical crystallographic axes.

In this work we address 3 specific questions: Where within the syncytia of the plumula do the initial mineral deposits begin to form? What is the initial mineral form, amorphous calcium carbonate or crystalline calcium carbonate, i.e. calcite? How do the proteins of the inter- and intrasyncytial spaces induce nucleation of the mineral and regulate its composition?

Materials and Methods

High Energy Microbeam X-Ray Scattering

In most previous studies of crystallinity, teeth were extracted from the urchin, washed, dried, and mounted for X-ray analysis, a process during which artifactual changes in crystallinity may have occurred. We avoided this by dissecting teeth from *Lytechinus variegatus* (Lv) and immediately placing each tooth in a 700- μ l microcentrifuge tube (internal diameter $\sim$ 4 mm) filled with

the released coelomic fluid and by performing room temperature, wide angle X-ray scattering (WAXS) with monochromatic 65.7-keV radiation (station 1-ID, the Advanced Proton Source, Argonne National Laboratory). Such high energy photons deposit very little energy in the specimen, minimizing beam damage, and furthermore allow the simultaneous collection of many more diffraction peaks than is possible with conventional energies (e.g. 8 keV) without the need for specimen oscillation. Millimeter or thicker specimens can be studied intact. In the first experiments, WAXS patterns were collected within 2.5 h of tooth isolation, but later experiments succeeded in collecting patterns within 40 min of extraction. The different stages of mineralization were examined by collecting in situ WAXS patterns from positions along the entire length of the tooth.

Scanning Electron Microscopy

Dried intact teeth were grossly fractured in large pieces at various points along a tooth to see the fracture surfaces created at different locations. Some pieces, with either the plate or interplate surfaces exposed, were mounted on scanning electron microscopy (SEM) pins and coated with a 6-nm layer of gold. These were examined in a Hitachi 3500 SEM in the high vacuum mode.

Conventional Histochemistry: Serial Sectioning

It is difficult to section a heavily mineralized object without fracturing the mineral and having the mineral chip out. It is also difficult to insure that the soft tissues are reached by the stains. This was overcome, in part, by fixing the tissues in vivo by placing an urchin in sea water (Instant Ocean) containing 0.1% in glutaraldehyde for 40 min. The teeth were dissected and immediately placed in fresh sea water-glutaraldehyde and held for 2 h at 4°C. They were then soaked overnight in Karnovsky's fixative at 4°C. After multiple washes in 0.1 M cacodylate to remove the excess fixative, the teeth were postfixed in 1% OsO₄ for 1 h. Solvent exchange continued with series of graded ethanol to 100%, then to 100% propylene oxide, and finally with 2:1 Epon/propylene oxide. After at least 4 h in this mixture, the teeth were embedded in 100% Epon and cured at 60°C overnight. The teeth were cut into blocks and then consecutively to 1- μ m sections. The first apical, aboral section of the plumula was labeled No. 1, and $\sim$ 2,800 sections in all were obtained. Because of the innate curvature of the teeth, the individual tissue blocks were oriented so as to present as perpendicular a cross section as possible. As a reference library, every 30th section was stained with toluidine blue [Veis et al., 1986, 2002, 2004]. The remaining sections were unstained and easily used for cross-polarization optical microscopy to determine crystallinity or for treatment with ethylene oxide to etch the surface Epon and expose epitopes for immunohistochemistry.

Protein and mRNA Isolation: Characterization of the Lv Proteome

The protocols for obtaining the mRNA and the mineral-related proteins, for cloning the relevant cDNAs and preparing the recombinant proteins, and for protein-specific antibodies have all been described in detail [Veis et al., 1986, 2002, 2004, 2007, 2009; Robach et al., 2006; Alvares et al., 2009].

Fig. 1. Development of mineral deposits within the plumula. **a** Plumula section 11, toluidine blue stained. The long blue arrow points to first mineral deposit. **b** Similar section, i.e. 31. The arrow points to the first mineral discernable as crystalline in cross Polaroids. **c–g** Serial sections 33, 35, 37, 39, and 43, respectively. Scale bar = 30 μm (**a**). All sections were taken and printed at the same magnification.

Results

Where within the Syncytia of the Plumula Does the Initial Mineral Begin to Form?

Märkel et al. [1969, 1986] and Kniprath [1974] showed very clearly that the plumula region is the site of origin of the initial mineralization and that the mineral deposits develop within membrane enclosed intracellular vacuoles created within the PO as they form the initial syncytia by coalescence of their cytoplasmic processes and the subsequent finger-like projections. The external membranes of the odontoblasts within a projection fuse to form a multinucleated syncytium. The intracellular internal cytosolic membrane-bound vacuoles, or mem-

brane sheaths, remain as enclosed compartments and provide the spaces, membrane surfaces, and matrix components that control mineral deposition. When the mineral plates are fully formed and mature, the interplate membrane may appear as a 4-layer membrane structure [Veis et al., 1986] when the mineral is removed.

Figure 1a shows an *Lv* plumula section cut 11 μm from the aboral end where the PO enter the epithelium covering of the plumula. Upon close inspection, 2 features are evident. First, in this toluidine-stained section, the PO are aligning and creating the individual syncytial layers. Second, the arrow in figure 1a shows where the first unstained mineral deposit is present, well removed from the epithelial layer. Based on the assumption that if the

Fig. 2. The 113 calcite diffraction spots from the plumula and adoral shaft of the living *Lv* tooth. The color bar (lower left) identifies diffracted intensities (log scale) [Killian et al., 2009]. The 2 patterns represent an approximately 2-mm difference in adoral position.

mineral was crystalline, it would be seen under crossed Polaroids, a number of serial sections were taken unstained and examined under crossed polarizers. Figure 1b shows that the initial site marked in figure 1a was indeed the location of a calcite crystal, and figure 1c–g depicts the increase in mineralization discretely along the syncytial layers. Figure 1c–g shows that the mineral phase grows exclusively from the most aboral part of the plumula along the syncytial layers and that no crystals have yet formed in the more adoral portion. It is of special importance that the calcite crystals appear simultaneously in adjacent syncytial spaces but without evident connections between calcite plates. This is consistent with the concept that crystals develop in separate intracellular membrane-bound compartments. Calcite can also be seen at several discrete positions within a single syncytial space and could reflect mineral nucleation at multiple independent sites or simply missing mineral pulled out during sectioning. Answering this question is a focus of current studies at a higher resolution. Synchrotron micro-CT of teeth in situ in the lantern and with $\sim 0.75\text{-}\mu\text{m}$ voxels failed to observe cross-syncytial links between adjacent primary plates or discontinuous mineral deposits [Stock et al., unpubl. data].

What Is the Initial Mineral Form, Amorphous Calcium Carbonate or Crystalline Calcite?

Recent papers [Beniash et al., 1999; Killian et al., 2009; Ma et al., 2009] have proposed that all sea urchin skeletal elements begin with the deposition of an amorphous precursor, i.e. amorphous calcium carbonate. Beniash et al. [1999] indicated that amorphous granules contain the calcium carbonate which transforms into calcite. Killian et al. [2009] proposed that it is the crystals of the mature part of the tooth that drive the crystallization and ‘single

Fig. 3. Toluidine blue-stained cross section of *Lv*, i.e. section 1,091, ~ 10 mm from the aboral end and ~ 20 mm from the adoral tip just at the point of keel formation. Scale bar = 1 mm.

crystal’ axes of the urchin calcite, possibly through the pillar bridges. However, as shown in figure 1, the first crystals form in the aboral plumula far removed from the adoral mature sections of the tooth and with no detectable mineral bridges to the mature crystalline regions. As described above, we took great pains to examine the mineral of the plumula in a ‘native’, living state by rapid and minimally degradative exposure of the plumula via direct examination using high energy synchrotron X-radiation. Figure 2 [Stock et al., unpubl. data] shows enlargements of the area around 113 calcite diffraction spots from the plumula, a tissue containing only primary plates and the rudiments of secondary plates, free of second stage (very high Mg calcite) densely packed mineral plates and far aboral from where the pillar bridges form. In the more mature adoral shaft, as expected [Stock et al., 2002], the single ring of the 113 diffraction peak and its complex substructure has been replaced with multiple rings; second stage mineral produces the arcs at different radii, reflecting the higher Mg content.

Fig. 4. Comparisons of toluidine- and anti-P16-stained *Lv* sections. **a** Section 1,091, toluidine blue. **b** Anti-P16-stained section 1,091. **c** Left corner of **b**. **d** Central section of **b** showing details of the developing keel. **e** Toluidine-stained section 2,784. **f** Anti-P16-stained section of **e**. Faint blue-DAPI nuclear stain in **c** and **d** marks the cell nuclei. The heavily mineralized primary plates do not fluoresce and appear black. Arrows indicate the position of the stone part at this incisal tip position. Scale bar = 1 mm (**a**, **b**, **e**). Sections **c**, **d**, and **f** are 3× magnifications of **a** and **e**, respectively.

How Do the Proteins of the Intersyncytial or Intrasyncytial Spaces Induce the Nucleation of the Mineral and Regulate Its Composition?

In the developing tooth, as in figure 3, where the tooth is still only moderately mineralized it has been shown at the transmission electron microscopy level [Märkel et al., 1969, 1986] that the mineral develops in membrane-bound sheaths directly aligned with either the syncytial (cell) membrane or the sheath (intracellular) membrane. In *Strongylocentrotus purpuratus*, Illies et al. [2002] and

Cheers and Etensohn [2005] noted that 2 low molecular mass proteins, i.e. P19 and P16, were involved in biomineralization. The P16 sequence had a predicted membrane-spanning sequence. We [Alvares et al., 2009] isolated the equivalent P16 from *Lv* and cloned, sequenced, and prepared anti-P16 antibody. The *Lv* P16 was shown to have a signal sequence along with a membrane-spanning sequence followed by a cytosolic retention carboxyl terminal domain. The extracellular sequence contained the typical Ser-Asp acidic sequence expected for a Ca-

Fig. 5. SEM. **a** Fracture surface in a mature adoral region [Stock et al., unpubl. data]. Scale bar = 30 μm . Note the primary plate midline material occluded in the primary plate calcite and the different texture of the secondary stage mineral fracture surface. **b** Fracture surface in a more aboral mineralizing region. Scale bar = 10 μm . # Developing column. * Thickening, full height interplate columns. **a** Lines point to the primary plate first stage mineral (high Mg calcite). **b** Lines point to the secondary stage mineral (very high Mg calcite).

binding site N-terminal to but near the membrane-spanning sequence. Anti-*Lv*P16 was used to localize the P16. As is apparent in figure 4, the anti-*Lv*P16 is directed at membranes, but this does not occur uniformly in all locations. In peripheral regions (fig. 4a–d) where mineralization is not evident, the syncytial cytosol and membranes are diffusely stained at the background level; the most intense staining is along the membranes in partially mineralized regions of active mineralization. The intensity of staining diminishes where mineralization is more complete and the intercrystalline syncytial layers become thinner. It is interesting to see (fig. 4b, e, f) that even at the heavily mineralized incisal edge of the tooth, including the stone part, there are still abundant cells and P16-stained membranes.

The high Mg calcite of the primary and secondary plates, needles, and prisms, called the first stage mineral, becomes cemented together by secondary stage very high Mg calcite. SEM of the fractured surfaces in a tooth cross section (fig. 5a) shows that in the adoral mature interplate regions the secondary stage calcite appears as islands growing within fenestrations of the interplate cellular syncytia. In the first stage mineral elements, the crystalline primary plates have fracture-related fragments or defect structures (fig. 5a) present as an interplate midline [Robach et al., 2005]. In the more aboral regions where the second stage mineral is developing the columns joining adjacent primary plates (fig. 5b), the columns develop independently, possibly from initially amorphous accretions of the very high Mg calcium carbonate or possibly directly as calcite. Secondary ion mass

spectrometry of cross sections of urchin mineral clearly relates the very high Mg calcite (30–40 mol% Mg) with a high content of an Asp-rich protein as compared to the lesser content in the first stage mineral [Robach et al., 2006].

Conclusions

The data described above allow us to conclude that mineral deposition in the *Lv* tooth begins within intra-syncytial membrane-bound compartments in the aboral plumula and is probably initiated by membrane-spanning proteins acidic in nature. Within the compartments the mineral deposition is nucleated simultaneously at multiple sites, again independently of each other. High resolution, high energy synchrotron X-ray scattering studies on living urchin tooth tissue show that the initial first stage primary plate mineral is crystalline calcium carbonate, that is, calcite. As depicted in the SEM in figure 5b, it is likely, in combination with the secondary ion spectroscopy data [Robach et al., 2006], that the initiation of the second stage mineral of very high Mg calcite depends on the presence of a protein distinct from that involved in the development of the first stage protein. The granular appearance of the earliest second stage mineral deposits is consistent with others observations of microstructures that they interpret as evidence for an amorphous stage of calcium carbonate that later transforms into oriented calcite.

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