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Sea urchin tooth mineralization: Calcite present early in the aboral plumula

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Abstract

In both vertebrate bone, containing carbonated hydroxyapatite as the mineral phase, and in invertebrate hard tissue comprised of calcium carbonate, a popular view is that the mineral phase develops from a long-lived amorphous precursor which later transforms into crystal form. Important questions linked to this popular view are: When and where is the crystallized material formed, and is amorphous solid added subsequently to the crystalline substrate? Sea urchin teeth, in which the earliest mineral forms within isolated compartments, in a time and position dependent manner, allow direct investigation of the timing of crystallization of the calcite primary plates. Living teeth of the sea urchin *Lytechinus variegatus*, in their native coelomic fluid, were examined by high-energy synchrotron x-ray diffraction. The diffraction data show that calcite is present in the most aboral portions of the plumula, representing the very earliest stages of mineralization, and that this calcite has the same crystal orientation as in the more mature adoral portions of the same tooth. Raman spectroscopy of the aboral plumula confirms the initial primary plate mineral material is calcite and does not detect amorphous calcium carbonate; in the more mature adoral incisal flange, it does detect a broader calcite peak, consistent with two or more magnesium compositions. We hypothesize that some portion of each syncytial membrane in the plumula provides the information for nucleation of identically oriented calcite crystals that subsequently develop to form the complex geometry of the single crystal sea urchin tooth.

Keywords

sea urchin; tooth; calcite; synchrotron radiation; x-ray diffraction; microCT; Raman spectroscopy

Introduction

Sea urchin teeth are fascinating, complex calcitic (crystalline calcium carbonate) structures and are an attractive model for biomineralization studies because each tooth grows and adds

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mineral continuously: Each tooth contains all stages of development. Aboral portions of the tooth (plumula, Fig. 1a) contain the early stages of mineralization and form plates of a “high” Mg calcite (10-20 mol% Mg) between layers of a highly organized cell syncytium. In the adoral direction beyond the plumula, the shaft (i.e., the tooth midsection) adds substantial mineral to the pre-existing calcite plates, cementing them together with “very high” Mg (>30 mol% Mg) calcite columns (also termed disks). The adoral (incisal) end completes mineralization. Despite many studies dating back to the 1800s (Salter 1861; Giesbrecht 1880), important unanswered questions about the growth of sea urchin teeth include: what specific proteins are involved, how do they control the calcium carbonate mineralization, what controls the change to the very high Mg calcite composition when columns are formed? Does calcite form directly from solution, as classical nucleation theory would propose, or as recent theories suggest via a transient amorphous solid-state phase? Whatever the mineral formation mechanism, at what stages of tooth development is crystalline material present and what dictates its orientation relative to the macroscopic orientation of the entire tooth (an orientation relationship repeated across the taxa of Echinoidea)? The fundamental question of where calcite exists in the growing tooth is addressed below using high-energy synchrotron x-ray scattering from living sea urchin teeth in their natural milieu. The present study demonstrates that calcite appears at the earliest stage of mineralization. High resolution Raman spectroscopy agrees, showing that an amorphous calcium carbonate (ACC) phase (if it exists) is below the limits of detection in the plumula. In the adoral, more heavily mineralized portions of the tooth (shaft and incisal end), Raman peaks are much broader and reflect the mixture of high and very high Mg calcite.

Mature sea urchin teeth contain well-organized plates, needles and prisms of high magnesium calcite $\text{Mg}_x\text{Ca}_{1-x}\text{CO}_3$ with $0.1 < x < 0.2$ (first stage mineral) and a cementing phase (second stage mineral) of very high magnesium calcite, $0.3 < x < 0.4$ (Schroeder et al. 1969; Stock et al. 2002). In the 1960s, debate centered on whether the mature, fully-dense tooth was a single crystal or not. Polarized light microscopy (Raup 1966) and x-ray diffraction (Nissan 1969; Donnay and Pawson 1969) found spine and test ossicles to be single crystals. The same optical techniques (Märkel and Titschack 1969; Märkel 1969) showed mature teeth were single crystals; powder diffractometry showed two Mg compositions (Schroeder et al. 1969); single crystal diffractometry showed the individual structural elements of teeth were single crystals (Berman et al. 1993; Wang et al. 1997) and microbeam x-ray diffraction mapping (Stock et al. 2002) of mature teeth showed the continuous ensemble diffracted as a single crystal with variable compositions (not as a highly-textured polycrystalline aggregate as in Al-Li alloys (Haase et al. 1999)). Early during tooth growth, however, each plate occupies a separate syncytial space parallel to the adjacent spaces, e.g. Stock et al. (2003), and remains a separate entity until the cementing phase is formed, both in juveniles (Devanesen 1922) and in adults (Salter 1861; Giesbrecht 1880). Later in tooth development, synchrotron microbeam diffraction (Stock et al. 2002; Ma et al. 2009) and secondary ion mass spectroscopy (SIMS) (Robach et al. 2006, 2009) showed that in the ensemble of first stage and cementing second stage mineral the plates are not only in contact but also have identical crystallographic orientations. One might argue that the mature tooth is a compositionally modulated crystal*, much like artificial “superlattices” grown by molecular beam epitaxy.

How then (and when) do the syncytia-surrounded plates in the immature sea urchin tooth come to have the same crystallographic orientations despite their physical separation? A recently proposed mechanism holds that the primary and secondary plates consist of solid

*Strictly speaking, the tooth is a bi-crystal with the elements on one side of the flange being slightly rotated with respect to those on the other side.

ACC, as reported in larval spicules (Beniash et al. 1997), in regenerating spines (Politi et al. 2004), and in the needles of the freshly dissected teeth (Ma et al. 2007), until pillar bridges (a very specific anatomic feature on a limited portion of the surface of the tooth) transfer crystallographic orientation from older to younger plates (Killian et al. 2009). This orientation transfer mechanism can be tested simply by recording diffraction patterns from the plumula, i.e., from the volume of the tooth without pillar-bridge connection to older (crystalline) plates. Such non-invasive interrogation of the plumula would reveal amorphous haloes if ACC were the only mineral phase present and sharp diffraction spots if the aligned (but spatially separated) plates contained calcite. Further, if multiple calcite crystals were present in the spatially separated spaces of the aboral plumula and their crystallographic orientations were identical and matched the mature tooth's orientation, then one could argue that the tooth's orientation is established by the younger, unlinked plates.

Thermodynamically metastable phases such as ACC may be very sensitive to disruption of their *in vivo* environment either by preparing specimens or by the actual process of observing the sample. The methods employed in the studies cited above studied teeth subjected to drying, embedding, sectioning, partial demineralization, long delays between dissection and observation, etc. which could induce crystallization where only amorphous mineral phases were present *in vivo*. The problem in using diffraction from the plumula to test the pillar bridge or other amorphous phase growth mechanisms, then translates into one of collecting the data while avoiding preparative artefacts and under speeds and conditions that minimally affect tooth tissue. All previous work of which the authors are aware suffer from the above sample preparation limitations which cannot be dismissed as unimportant.

The present report studies mineralization and crystal organization within the aboral plumula using synchrotron microbeam diffraction, i.e., from volumes that should only contain ACC if the pillar bridge mechanism were valid. The teeth (*Lytechinus variegatus*) were freshly dissected and remained in their epithelial membranes and in coelomic fluid, their native environment. Use of high energy x-ray diffraction limits the dose applied per diffraction pattern collected and, through the relatively large diameter of the Ewald sphere (see below), allows diffraction peaks from the arbitrarily aligned tooth to be collected at the first application of the beam to the tissue. Synchrotron microComputed Tomography (microCT) provided 3D microstructural data for correlation with the diffraction patterns. Raman spectroscopy also demonstrated calcite was present in the plumula and, in the adoral portions of the tooth, calcite peaks were broader, consistent with the expected multiple Mg compositions.

Materials and Methods

Teeth were dissected from a living adult *L. variegatus* sea urchin following protocols approved by Northwestern University's Animal Care and Use Committee. One live tooth (tooth 1) was immediately placed into a 700 μ l microcentrifuge tube (internal diameter $\sim$ 4 mm) filled with coelomic fluid from the same animal and kept at ambient temperature. A second tooth (tooth 2) from the same lantern was immediately plunged into liquid nitrogen; after several minutes it was placed into a matching tube but without fluid. In addition, a lantern was dissected from a second animal and was cast in LR White (Electron Microscopy Sciences, Hatfield, PA). A tooth from a third animal (tooth 3) was recovered from the bottom of the aquarium, rinsed in tap water and dried. Teeth 1 and 2 were $\sim$ 30 mm in length; tooth 3, missing the plumula, was about $\sim$ 20 mm in length.

The diffraction patterns of teeth 1 and 2 were collected at station 1-ID of the Advanced Photon Source (APS) using the experimental arrangement shown in Fig. 2. A beam of 65.7 keV x-ray photons and of dimensions 100 μ m vertical $\times$ 50 μ m horizontal diffracted from

each specimen and was recorded with a General Electric Angio amorphous silicon detector, a medical X-ray imaging detector designed for high-energy X-rays (80 keV), for high contrast (14-bit) and for rapid readout. The detector of $(2K)^2$ pixels, each $200\ \mu\text{m} \times 200\ \mu\text{m}$, consisted of a $500\ \mu\text{m}$ CsI scintillator layer deposited on top of an amorphous Si TFT (thin film transistor) array. Each pattern was collected by integrating for 1 s. Diffraction patterns from a ceria reference (CeO_2 , NIST SRM-674a) calibrated the area detector-specimen separation ($\sim 1\ \text{m}$), beam center and detector tilts ($\sim$ perpendicular to the incident beam).

Because x-ray irradiation might initiate crystallization of mineral that was entirely amorphous, the dose delivered to the specimen was kept to a minimum. This means diffraction patterns were recorded from the arbitrary orientation in which the “crystal” tooth was placed in the beam, and the sample was not rotated to bring “more convenient” or more intense *hkl* into the Bragg condition.[†] Prior experience with diffraction of *L. variegatus* teeth (Stock et al. 2002) and consideration of the high energy Ewald sphere diameter and the density of reciprocal lattice points for this hexagonal crystal (see Discussion, Fig. 7) indicated that several strong reflection would be recorded regardless of sample orientation.

Synchrotron microCT was performed at station 2-BM, APS, on a tooth in the intact lantern and on tooth 3. For the tooth in the intact lantern, a local tomography approach was used, where only the region-of-interest remains in the field-of-view of the tomography detector and the rest of the sample is ignored as it moves into and out of view. The image of the primary plates in the plumula was produced using the $1.5\ \text{mm}$ diameter volume surrounding the plumula of one tooth within the intact lantern cast in plastic ($\sim 9\ \text{mm}$ diameter). Projections were recorded every 0.12° and with 25 keV photons. Reconstruction was with $0.75\ \mu\text{m}$ voxels. Tooth 3 was imaged with 20.9 keV photons, projections were recorded every 0.125° and the reconstructions were $(2K)^2$ with $\sim 1.8\ \mu\text{m}$ isotropic voxels.

Raman microprobe spectroscopy was carried out in the Keck II Laboratory at Northwestern University using a Princeton Instrument/Acton TriVista CRS Confocal Raman System. Samples were prepared by two separate methods for two separate analyses with the Raman microprobe. The first analysis surveyed crystallinity along the length of the tooth using powdered sections, and the second probed the transverse (axial) cross section of the tooth. Spectra were recorded over the range $115\ \text{cm}^{-1}$ to $1600\ \text{cm}^{-1}$, using a laser excitation of $514.5\ \text{nm}$. Spectra were taken at magnifications of $10\times$ ($50\ \mu\text{m}$ spot size) and $100\times$ ($5\ \mu\text{m}$ spot size), with the larger spot size yielding a better average of the sample composition. The wavenumbers of the observed bands were not corrected to an emission standard, but the band at $\sim 1122\ \text{cm}^{-1}$ (due the florescent lights present in the laboratory) served as an internal standard; given an excitation wavelength of $514.5\ \text{nm}$, this band should be Raman active at $1125\ \text{cm}^{-1}$ (Settle, 1997).

In the first analysis, 15 teeth were extracted from three sea urchins (mass: 77.75 g, 76.05 g, and 110.94 g). The plumula of each tooth was divided into two sections (cut transversely) to create sections (from aboral to adoral) P1 to P2 and section P2 to S1 (see Fig. 5). The remaining portion of the tooth was divided into three sections of 6 mm length, cutting from the adoral end to the aboral end, S4 to S3, S3 to S2, and S2 to S1, respectively. In each of the three adoral sections, the flange was separated from the keel using cuticle clippers. The corresponding sections of the 15 teeth were pooled and flash frozen using liquid nitrogen. Each group of flash frozen sections was then milled in a freezer mill (Spex Model 6700) under liquid nitrogen for 3 min. The powders of each group of sections were collected and any powder residue within the freezer mill vial was rinsed out with 100% absolute anhydrous Ethanol (at $-80^\circ\ \text{C}$) and added to the powdered samples to assure as much as

[†]Note the use of Miller-Bravais indices used here and below for this hexagonal crystal system, e.g., Cullity and Stock (2001).

possible of the original tooth material was collected. All powdered samples were kept dry under desiccant in a -80° C freezer until analysis 24 hr after harvesting.

In the second analysis, teeth were extracted from a single sea urchin (mass 86.91 g) and the wet tooth was immediately embedded in LR white (Electron Microscopy Sciences, Hatfield, PA), 7 mL of LR white to 1 drop of accelerant, to encase the fresh tooth within 15 minutes of extraction. After 15 min curing, the most aboral end of the embedded tooth was cut flush (transversely) with a glass knife on a Reichert-Jung Ultracut E Ultramicrotome, to reveal the most aboral tip of the plumula. The end of the embedded tooth was then cut from the rest of the block as a 2 mm thick section, with a fine tooth coping saw, and the section was mounted on a glass slide with the aboral tip of the plumula facing up. The tooth section was then examined with no further preparation.

Results

MicroCT, overall plumula structure

Regular sea urchins such as *L. variegatus*, the subject of the present study, possess a jaw structure (Aristotle's lantern) with five pyramids arranged around a common axis. Each pyramid contains a single tooth (Fig. 1b). In *L. variegatus*, the mature tooth cross-section is T-shaped; the leg is termed the keel and the bar across the top is called the flange. The flange forms in the plumula, and development of the keel marks the transition from the plumula to the shaft. Figure 3 of tooth 3 shows a series of synchrotron microCT slices from positions before the keel formed to nearly complete mineralization; only one-half of the flange is shown. Slice A (Fig. 3a) shows developed primary plates (pp) and the beginnings of secondary plates (sp). At a position 2.00 mm adoral of A (slice B, Fig. 3b), the primary plates retain the same lateral dimensions, the secondary plates enlarge considerably and the needle-prisms (np) are visible. The next 4.00 mm (slices C, D) brings development of carinar process plates (cpp) and early stages of keel formation (K); the flange (F) expands considerably, nearly reaching its full lateral extent. Examination of multiple slices (to either side of the slice shown in set D) at highest magnification reveals only the ends of the primary plates on the abaxial surface (ab) of the flange and no evidence of pillar bridges. At 8.00 mm from slice A, the keel is close to its full length (cropped from panel E), and second stage mineral is not yet visible linking first stage mineral structures such as the prisms, carinar process plates and secondary plates. At 10.00 mm (slice F), the plates are largely cemented together by the very high Mg columns (second stage mineral), and, aside from further densification, the tooth cross-sections G-J change little. The insets of slice E, G and I trace the development of pillar bridges on the abaxial flange surface. In slice I, one sees well-developed pillar bridges, and none are present in the more aboral images: the matching area of slice G is smooth. Thus, pillar bridges appear only many millimeters from positions producing diffraction from discrete primary plates (see below, Fig. 4b, c).

In the plumula, primary plates are the first mineralized structures to form, and the 3D image of the most aboral portion of plumula (Fig. 1c) shows two pairs of primary plates with the intervening fifty plates rendered transparent. The gold pair are more mature (larger, curved and overlapping) than those in blue (PP1 and PP2, smaller and more planar). Because the pillar bridges form only adoral of the plumula-shaft transition on the abaxial surface of the flange (Märkel 1969), i.e., the very top of the "T" as shown in Fig. 2, comparing diffraction patterns from the plumula with those from the shaft and the incisal end directly determines when calcite forms and whether its orientation continues throughout the tooth's length.

X-ray diffraction

Within 2.5 hrs of dissection, high-energy x-ray diffraction patterns were being collected from the living tooth in coelomic fluid. Figure 4 shows diffraction patterns from the living tooth in coelomic fluid. The red arrows adjacent to the schematic of the tooth (Fig. 4a) show where the specific numbered diffraction patterns were collected. Patterns 20-27 were from the plumula, patterns 28 and above were from the shaft and the green arrow marks the approximate beginning of the incisal region. One quadrant of diffraction patterns typical of the plumula is reproduced in Fig. 4b. The polycrystalline rings marked with asterisks are from the microcentrifuge tube. Single-crystal calcite diffraction spots are clear, e.g., the 11.6 spot enclosed in the black rectangle. Complete or even broken calcite diffraction rings, characteristic of polycrystalline specimens, are absent. Figure 4c shows the enlarged 11.6 spot from pattern 21, the aboral portion of the plumula; this pattern was recorded without prior exposure of the tooth to x-radiation. Figure 4d shows an enlargement of the 11.6 spot from pattern 28, a position where the keel and second stage mineral (very high Mg calcite) have begun to form. Corner-to-corner, Fig. 4d spans 12° azimuthally and Fig. 4c somewhat less.

In Fig. 4c and d, two groups of discrete diffraction spots comprise the tooth's 11.6 reflection. One set is from PP1 and the second from PP2, i.e., the two stacks of plates identified in Fig. 2b. The centers of the groups of spots are 7-9° apart azimuthally; this is in line with earlier observations (Ma et al. 2009). These discrete spots are from individual primary plates within the two stacks, slightly misoriented from each other, and the rotations between adjacent reflections are a fraction of a degree. It is difficult to tell precisely how many spots comprise the reflections from PP1 and PP2 because they are so close together and vary so much in intensity, but Fig. 4c shows at least 5-8 spots in PP1 and 11 or more in PP2. The discrete spots of the 11.6 reflection of Fig. 4c (both PP1 and PP2) are all at same radius from the beam center and correspond to a single composition $x \sim 0.13$ for $\text{Mg}_x\text{Ca}_{1-x}\text{CO}_3$.

Figure 4d shows the 11.6 reflection where the keel and second stage mineral are present: solid arcs are seen for each of the four calcite compositions (radii), not discrete resolvable spots within each arc. Similar solid arcs with multiple sets of spots were seen in *L. variegatus* previously (Stock et al. 2002) and in *Paracentrotus lividus* (Ma et al. 2009). The arcs of spots in Fig. 4d span a composition range $\Delta x = 0.35$, somewhat larger than reported previously with synchrotron microbeam x-ray diffraction in *L. variegatus* (Stock et al. 2002) but slightly less than the 0.40 range in reported for *L. variegatus* by Schroeder et al. (1969).

Fewer diffraction patterns were recorded of the flash frozen *L. variegatus* tooth than of the living tooth. Figure 4e shows the 12.2 diffraction spot from the flash frozen tooth; this pattern is from a tooth position similar to that in Fig. 4c and is somewhat enlarged compared to the pattern in 4c. The patterns from the frozen tooth do not differ appreciably from those of the living tooth except for a difference in tooth orientation relative to the x-ray beam direction (see Supplemental Fig. 1).

Raman spectroscopy

The Raman spectra of the powdered preparations of sequential segments of the flange from adoral incisal segments S4-S3, S3-S2, S2-S1, and the aboral plumula segments P2-S1 and P1-P2 at the most aboral end, are shown in Fig. 5 along with the spectrum of the standard geologic calcite. These large samples provide the averaged composition of each region. The characteristic Raman active modes of interest are the 154 cm^{-1} [translation, T] and 281 cm^{-1} [libration/rotation, L] lattice vibrations of the calcite crystal, and the 711 cm^{-1} ν_4 in-plane C-O bending, and 1085 cm^{-1} ν_1 symmetric C-O stretch carbonate modes. Loss of the diagnostic crystal translation and libration modes are often a metric used when determining

the presence of ACC using Raman spectroscopy. These crystal lattice modes produce sharp peaks in the calcite standard (Fig. 5f). These same modes are prominent in spectra of the aboral plumula (Fig. 5e,d) but are lower in intensity and broader. The crystal lattice peaks continue to widen as one advances adorally in the more mature parts of the tooth and become almost undetectable peaks near the incisal end.

Fig. 6 shows direct observation via the Raman microprobe of the surface of a microtomed transverse section through the intact plumula which had been immediately encased in a rapidly setting LR White plastic without any further treatment, as described. The most aboral position possible was sampled at 5 discrete spots traversing the plumula from one side to the other, and, as a control, the LR white outside the plumula. These spectra were collected at the same resolution and collection times as the powdered samples of Fig. 5.

Discussion

X-ray diffraction

The diffraction patterns from the *L. variegatus* teeth demonstrate that calcite is present at the earliest stages of mineralization, but several questions must be considered. First, is the number of spots within each of the reflections consistent with the number of discrete primary plates within the irradiated volume? Second, does the dissection process disturb the local environment enough to cause calcite to form from a non-crystalline precursor? Third, does x-irradiation (and/or any accompanying temperature rise) cause crystallization that would otherwise not occur until much later in tooth growth?

The primary plates are not flat but lie parallel to one another. Near the aboral end of the plumula (Fig. 1c), the spacing normal to the plate surfaces is $\sim 3.7 \mu\text{m}$ center-to-center, with slightly more empty space than calcite. At the position shown in Fig. 4c (pattern 21), the plates make an angle $\sim 45^\circ$ with respect to the slice plane and $\sim 10^\circ$ from perpendicular to the tooth's longitudinal axis. With respect to the microbeam direction, the primary plates were also inclined by $\sim 45^\circ$, and the $100 \mu\text{m}$ wide beam (vertical) intersected at least 55 primary plates (more if the tooth were more tilted). About 20 discrete spots comprise the 11.6 diffraction peaks in Fig. 4c, not 55 or more. Therefore, not every irradiated plate is oriented for diffraction, and this is not surprising given that, unlike in the shaft region, the plumula is highly curved, rotating nearly 180° over less than 5-10 mm length (Fig. 2a). MicroCT of the *in situ* plumula shows that the plates rotate to accommodate this trajectory, a rotation that is accommodated by slight deformation of each cellular syncytium separating the primary plates (data not shown). The tooth was straightened to fit in the tube (a single discrete break mid-plumula), but the curvature remained in the two sections, and one would expect only a fraction of the primary plates diffract for one incident beam direction, even though the Ewald sphere diameter is quite large at this energy, Fig. 7, compared to that at 8 keV (Cu K α radiation).

Diffraction patterns of the *L. variegatus* tooth described above were collected beginning 2.5 hr after dissection. The large Ewald sphere diameter and dense reciprocal lattice net for calcite means that some *hk.l* are always oriented for diffraction (Fig. 7); crystal orientation is needed only if a specific reflection is required, see Cullity and Stock (2001). This time (completion of dissection to acquisition of the first diffraction pattern) has been reduced to about 0.5 hr in subsequent experiments on additional teeth, without changing the diffraction results. Given that the teeth were immediately returned to coelomic fluid after extraction and that the teeth remained moist and within their membrane throughout, it is unlikely that the environment induced crystallization of ACC precursors: needles (from the stone part of sea urchin teeth) were reported to require more than 8 hr in pure water to begin to exhibit birefringence (Ma et al. 2007); i.e., to transform from ACC to calcite, and the authors are

unaware that another group has replicated this finding. Note also that calcite is ~ 100 fold more soluble in sea water than in pure water (Mucci 1983); any transition from ACC to calcite in sea-water-like coelomic fluid is probably slower than in pure water due to the difficulty in forming calcite in such a solution (but this may not be relevant because the mineral is enclosed in syncytia and epithelium and not directly exposed to the surrounding fluids except as they affect the syncytia).

The intense x-ray beam possibly could nucleate calcite from precursor ACC either by heating the specimen or by radiation-induced transformation. The monochromatic x-ray microbeam used in these experiments delivers heating equivalent to that of a laser pointer, a temperature rise of less than 1°C . Heating-induced transformation of ACC, therefore, can be discarded as a source of crystallization. As calculated from the NIST x-ray absorption tables (Hubbel and Seltzer, 2010), the local dose was 300-400 Gy per diffraction pattern. Although such a dose could affect cell activity, it is unlikely to nucleate calcite from ACC, given, as noted above, the transformation requires > 8 hr exposure to pure water (as opposed to the natural environment of coelomic fluid). Further, a monolayer of amorphous calcium carbonate on an organic layer did not crystallize after many hours exposure to synchrotron radiation at doses one can infer were much higher than in the present experiments (DiMasi et al. 2006). At room temperature protein crystals remain stable at doses higher than 10 kGy (Rajendran et al. 2011). Feldman et al. (2009) crystallized amorphous barium titanate thin films; ignoring the much greater absorption of these samples at 24.3 keV (than in the sea urchin calcite at 65.7 keV), the exposure of $\sim 7 \times 10^8$ photons/ μm^2 was over 230 times that in the experiments reported above. Martis et al. (2011) found thin films of lithium disilicate at 450°C did not crystallize under 10 keV irradiation at exposures of $\sim 4 \times 10^7$ photons/ μm^2 (0.5 hr) and only partially crystallized at 600°C after 4 hr exposure.

The small lateral dimensions of the calcite primary plates in the irradiated volume have an important implication vis-à-vis the stability of ACC (if any is present). Tooth growth rates in related sea urchins are calculated to be on the order of $10 \mu\text{m/hr}$ (Robach et al. 2009) based on data in the literature (Märkel 1969; Holland 1965), i.e., three plates are added per hour. For this growth rate, the 55 plates estimated to be in the irradiated volume represent a temporal snapshot of ~ 20 hr. The results suggest that pure ACC (absent any calcite) cannot persist more than hours after mineralization begins. To be more definitive, the microCT and diffraction data need to be from the same volume of the same tooth and registered within a few tens of micrometers.

Calcite single-crystal patterns are observed both from flash frozen and from living (dissected) teeth, and this result supports the conclusion that the tooth has calcite at its earliest stage of mineralization. Rapid freezing and large under-cooling should produce many nucleation sites in an ACC precursor phase. If the primary plates consisted solely of ACC at the point when the teeth were dissected and transformed to calcite subsequently, it is unlikely that externally induced nucleation could produce calcite single-crystal diffraction patterns.

It is possible that crystallographic orientation may be transferred from plate to plate via very narrow interconnections running through the cellular syncytia; these would be absolutely different from the pillar bridges. If such interconnections existed, there would probably be very few per plate, and the odds of finding an interconnection in a TEM thin section would be vanishingly small, so synchrotron microCT remains the best prospect for locating any such interconnections within the plumula. The intact aboral plumula in Fig. 1c was imaged with $\sim 0.75 \mu\text{m}$ isotropic voxels; this is the highest resolution, noninvasive data available but the reconstruction is quite noisy because of the local tomography approach. In Fig. 1c, therefore, one might not detect sub-voxel interconnections, but reconstructions of dissected

aboral plumulae of *L. variegatus* (not shown, with 0.75 μm or with 1.45 μm voxels and with much better signal-to-noise levels than in Fig. 1c) also fail to reveal trans-syncytial interconnections between adjacent primary plates when they would be expected to detect such mineral features with minimum dimensions one-quarter of the voxel size (Stock 2008). Even if interconnections do exist, one is still left with the question of what nucleated the first mineral when the first tooth plate formed in the juvenile urchin and why it always takes the same orientation.

The data and the above considerations suggest, therefore, that there is a single, independent nucleation site for each primary plate. It is reasonable to hypothesize that some portion of the syncytial membrane nucleates the calcite of the primary plates; this as-of-yet-unidentified structure is found in every syncytial space of the tooth, perhaps at many places within a single space, but nucleation only occurs at one place and produces a highly specific calcite crystal orientation. Alvares et al. (2009) have presented evidence that the sea urchin tooth protein UTMP16, is a membrane-anchored protein probably presenting an aspartic acid - serine rich sequence (GGSDTSSDTGSDDDSIDDDGSSDDSS) on the external side of the urchin tooth syncytial space and may be responsible for the mineral nucleation, similar to the proposal that the Asprich 2 peptide sequence (DEADEADADEADADEADADNDAADETAADVGTAEADVADDE) (Gotliv et al. 2005) may nucleate calcite on surfaces (Metzler et al. 2010). Although the present x-ray scattering data cannot address whether ACC is injected into this space from vacuoles and rapidly crystallizes (or persists in contact with the calcite) or whether calcite grows directly from a mother liquor, the data clearly demonstrate that crystalline calcium carbonate (calcite) is present and originates in earliest stages of urchin tooth mineralization. The fact that each primary plate independently takes the same crystallographic orientation is a strong albeit indirect indication that crystallization in the sea urchin tooth is a tightly controlled process although the shape of elements such as primary plates is dictated by the spatial constraint of the syncytial space in which they grow.

The single crystal diffraction patterns presented here offer no guidance as to the amount or even presence of ACC in the plumulae of *L. variegatus*. There are too many amorphous scatterers present in the beam (tube containing the specimen, soft tissue in the tooth, coelomic fluid). The goal was the opposite, to determine how early in tooth mineralization calcite could be detected in living teeth, and the experiment was designed with this in mind, not hunting ACC, a task fraught not only with difficulty in terms of scattering experiments from small amounts of material but also in terms of ambiguity of interpretation.

Raman spectroscopy

One advantage of Raman spectroscopy is the distinct signature of ACC. If there is ACC in the plumula, it should be clearly evident unless its concentration is below detection limits. The Raman spectra of the powdered preparations of sequential segments of the flange and of the geologic calcite standard (Fig. 5) provide the averaged composition of each region. The characteristic Raman active modes appeared clearly in the geologic calcite, specifically, the 154 cm^{-1} [translation, T] and 281 cm^{-1} [libration/rotation, L] lattice vibrations of the calcite crystal, and the 711 cm^{-1} ν_4 in-plane C-O bending, and 1085 cm^{-1} ν_1 symmetric C-O stretch carbonate modes. The diagnostic crystal translation and libration modes also are prominent in the spectra of the aboral plumula (Fig. 5e,d), but diminish in intensity and peak sharpness as the more mature parts of the tooth are reached and become almost undetectable in the adoral flange.

In Fig. 5, the major carbonate peak at 1085 cm^{-1} also diminishes in intensity, broadens and shifts slightly to higher wavenumber as one moves adorally from the plumula to the incisal end, consistent with the observations of Wang et al (2012) on effect of Mg composition on

peak position. The broad ACC band seen in the region between 150 to 250 cm^{-1} in authentic ACC (Urmos et al. 1991; Raz et al., 2002; Weiss et al., 2002; Weiner et al., 2003; Jacob et al. 2008; Neues et al., 2011; Wehrmeister et al., 2011) is not present in Fig. 5, confirming the crystalline nature of the calcite in the plumula. The broadening of the peaks at 154 and 281 cm^{-1} in the plumula spectra (Fig. 5d,e) as compared to geologic calcite (Fig. 5f) suggest that, according to Urmos et al. (1991), the plumula calcite contains ~ 10 mol % Mg. The shift of the ν_1 carbonate band to higher frequency also speaks to the increasing content of Mg calcite in the more mature parts of the tooth and argues against the presence of increasing ACC because the carbonate frequency in ACC is near 1079 cm^{-1} (Urmos et al., 1991; Wang et al. 2012); this shift and peak broadening is also consistent with the increasing amount of second stage calcite that occurs with tooth maturation, i.e., increasing ratio of second stage calcite, $x \sim 0.33$, to first stage calcite, $x \sim 0.13$, found in *L. variegatus* (Stock et al. 2002).

The microtomed transverse section through the intact plumula was sampled at five discrete positions and within the LR white outside the plumula and showed no evidence of amorphous material (Fig. 6). At each position, the calcite bands at ~ 154 and 281 cm^{-1} were clearly observed. Although these spectra were collected at the same resolution and collection times as the powdered samples of Fig. 5, more spectral details (Raman active modes) appeared in these spectra. This higher resolution of more Raman active modes is likely due to the nature of the sampled material. While the first preparation, by design, is meant to examine a random homogenous powdering of relatively large sections of the tooth, the second preparation is a direct observation of an intact cross section of the urchin tooth with the underlying architecture and distribution of material kept intact. The LR White spectrum, Fig. 6a, collected fewer counts over a longer time period, had a much lower Raman emission than the plumula surface spots, confirming that the tooth sections being sampled were not contaminated by the embedding medium. The most notable peak observed in sampling of LR White, at 1122 cm^{-1} , and visible in all other spectra in this figure, was an artefact due to the florescent lights present in the laboratory (Settle, 1997).

From the aboral plumula to the adoral incisal end (Fig. 5), peak intensities decreased while the peak widths increased. Based on the work of Long et al. (2012), it is most likely that this apparent change in relative “crystallinity” is related to the increased content of Mg, but this has yet to be specifically determined. If several populations of spatially discrete calcite with different Mg contents were present within the sampled volume, the respective peaks would be slightly displaced and be observed as a broadened peak. In Fig. 6 the Raman spectra sampled from 5 points along the transverse cross section of the plumula confirms (via the presence of the translation and libration lattice modes) that the most aboral tip of the plumula is calcitic with no sign of ACC present (within the resolution of the microprobe). What is also clear from the Raman analysis of the data along the length of the tooth, in confirmation of the X-ray data, the plumula of the sea urchin tooth (aboral to the pillar bridges) is calcitic with no sign (within the resolution of the microprobe) of amorphous material present.

Comments

The key aim of this work was direct analysis for the presence of calcite within the complex multi-structured and compartmentalized sea urchin tooth under conditions where amorphous calcium carbonate (ACC) might also be present *in vivo*. Synchrotron x-ray diffraction on the living tooth within its natural environment (intact epithelial membrane surrounded by coelomic fluid) detected crystallinity without the complication attendant upon tissue preparation. It is important to emphasize that the diffraction experiments are the least invasive interrogation of the tooth plumula reported to date. It was difficult to carry out the confirmatory Raman analyses, which could distinguish between ACC and calcite, without

tissue isolation prior to sampling. As described above, the Raman analyses were completed within a few hours, under conditions permitting minimal conversion of any possible ACC to calcite. The resulting Raman spectra provided no evidence for the presence of ACC in the aboral plumula region where mineral formation at $x \sim 0.1$ begins. In the more mature portions of the tooth, containing mineral phases with Mg contents from $x \sim 0.1$ to $0.30 < x < 0.40$ (Schroeder et al. 1969; Stock et al. 2002), the spectra showed broader, less intense peaks consistent with heterogeneous composition and quantities of very high Mg second stage mineral as one moved adorally.

Plants and animals have evolved a myriad of biomineralization strategies. Bone of vertebrate animals, a composite of calcium phosphate nanoplatelets and of collagen (Weiner and Wagner 1998; Currey 2002), has received considerable attention because of its importance in human health. The bone analogs (ossicles) of sea urchins and other echinoderms (invertebrates) are predominantly calcium carbonate in the form of calcite crystals of enormous geometrical complexity. Despite the different mineral bases, the proteins associated with mineral in bone and in sea urchin ossicles have remarkable commonalities, tending to be highly acidic and phosphorylated and to have similar sequences (Alvares 2009). The study of sea urchin mineralization provides a very valuable window into aspects of vertebrate bone and tooth formation but has great intrinsic worth in its own right.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Tooth geometry, Aristotle's lantern and the aboral plumula of *Lytechinus variegatus*. a. Tube-based microCT 3D-rendering of a single *L. variegatus* tooth. The aboral (abo) and adoral (ado) directions are indicated. Following Märkel (1969), the length of the dotted lines indicate the extent of the plumula, shaft and incisal end. In terms of anatomical (mineralized) landmarks, the plumula extends until the keel begins to form (see Fig. 3). Once the keel starts to develop, the structure is termed the shaft and the pillar bridge structure begins to form (Märkel 1969). Where the tooth passes beyond the dental slide (see b.), the pillar bridges disappear and this landmark designates where the incisal end begins. b. 3D rendering of the mineralized tissue of a lantern produced with tube-based microCT. The portion of the lantern closest to the viewer was numerically excised to expose one tooth's keel, flange and dental slide (where collagenous tissue connects to the tooth's pillar bridges). The plumula is identified with the dashed red line. c. Synchrotron microCT of the aboral portion of the plumula imaged *in situ* from a small adult lantern cast in polymer. The arrows from a. show the approximate position of the slices. Two pairs of primary plates are shown in 3D; the upper pair (gold in the on-line version) which overlap at their center is more mature than the lower pair which do not overlap (labeled PP1 and PP2 and blue in the on-line version). The gray-scale image at the back is a numerical section approximately normal to the plate pairs; the lighter the pixel, the more attenuating the volume element (voxel). The white arrow marks the centerline of the tooth. (Online version in color).

Figure 2. Experimental set-up at station 1-ID, the Advanced Photon Source, for recording high-energy x-ray diffraction patterns from *L. variegatus* teeth on a 2D detector. The tube containing each tooth is not shown, and η is the azimuthal angle in the diffraction pattern.

Figure 3.

Portions of slices of a *L. variegatus* tooth from positions considerably adoral from the position shown in Fig. 1b (5-10 mm away). Each image is 2.00 mm adoral from the preceding image and showing one-half of the flange. The scale bar is 0.5 mm. The vertical field-of-view (FOV) of inset areas is 180 μm , and the arrows locate the areas enlarged. The labels are explained in the text.

Figure 4.

a. Tube-based microCT 3D-rendering of a single *L. variegatus* tooth. Red arrows mark approximate positions where patterns 20, 21, 27, 28 and 33 were recorded ($y = -23.6, -19, -15, -13$ and -3 mm, respectively), and the green arrow marks the approximate division between shaft and incisal end. The black, double-headed arrow shows the aboral (abo) and adoral (ado) tooth directions. b. One quadrant of diffraction pattern from position 21. The beam stop is at the lower right; “*” mark diffraction rings from the semi-crystalline polymer of the tube containing the tooth and/or from the coelomic fluid (these rings are present when the beam traverses the tube plus coelomic fluid without encountering the tooth); the black box encloses the 11.6 calcite diffraction spot. Quadrant of $(1024 \text{ pixels})^2$, color bar in units of 10^2 cts. c. 11.6 diffraction spot of position 21, typical of the aboral plumula. 140 detector pixels horizontal FOV, color bar in units of 10^5 counts. d. 11.6 diffraction spot from location 28 which was 2 mm adoral from last observation of a single small arc of spots. 140 detector pixels horizontal FOV, color bar in units of 10^2 counts. e. 12.2 diffraction spot from the flash frozen *L. variegatus* tooth from a position comparable to that where pattern 21 (c) was recorded. The 12.2 spot was from the quadrant opposite that in b.,c. 100 detector pixels vertical FOV, color bar in units of 10^2 counts. (On-line version in color).

Figure 5.

Raman spectra of powdered samples of the flange. a-e. show patterns from the most adoral (incisal tip) to the most aboral (plumula), respectively. The regions collected for analysis are illustrated graphically at the right; the labels are identified in the text. f. shows powdered geologic calcite as a reference. Raman active bands T (translation), L (libration), ν_4 (in-plane bending), ν_1 (symmetric), and ν_3 (antisymmetric) modes have been marked. All six spectra were plotted over the same range of wavenumbers. The relative height of a. and b. are magnified by 120% to better show the relevant points of interest, while plot e. was reduced to 80% and plot f. was reduced to 70% of full height.

Figure 6.

Raman spectra collected from a. the LR white embedding material and b.-f. 5 points (50 μm spot size) across the most aboral tip of the plumula of a whole sea urchin tooth. The spectrum of each point clearly shows the presence of the translation ($\sim 154 \text{ cm}^{-1}$) and libration ($\sim 281 \text{ cm}^{-1}$) lattice modes of calcite. Since the sample is not perfectly flat, the microscope image of the most aboral tip of the plumula is a patchwork composition (denoted by dashed lines) of various points along the tooth that have been brought into focus in order to obtain the Raman spectra.

Figure 7.

Ewald sphere construction for the calcite (10.1) reciprocal lattice plane and 8 keV vs 65 keV photons, see Cullity and Stock (2001). The large circle around the 00.9 reciprocal lattice point indicates schematically how imperfect (strained, mosaic-containing) crystals have a much broader diffraction peaks and can produce significant intensity rather far from the exact diffraction conditions.