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Calcite orientations and composition ranges within teeth across Echinoidea

Stuart R. Stock¹, Konstantin Ignatiev^{1,*}, Peter L. Lee^{2,†}, and Jonathan D. Almer²

¹Department of Molecular Pharmacology and Biological Chemistry, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA

²The Advanced Photon Source, Argonne National Laboratory, Argonne, IL, USA

Abstract

Sea urchin's teeth from four families of order Echinoida and from orders Temnopleuroida, Arbacioida and Cidaroida were studied with synchrotron x-ray diffraction. The high and very high Mg calcite phases of the teeth, i.e. the first and second stage mineral constituents, respectively, have the same crystallographic orientations. The co-orientation of first and second stage mineral, which the authors attribute to epitaxy, extends across the phylogenetic width of the extant regular sea urchins and demonstrates that this is a primitive character of this group. The range of compositions x for the two phases of $\text{Ca}_{1-x}\text{Mg}_x\text{CO}_3$ is about 0.20 or greater and is consistent with a common biomineralization process.

Keywords

Biomineralization; calcite; sea urchin; synchrotron radiation; teeth; x-ray diffraction

Introduction

Echinoderms have an endoskeleton of calcite, the Echinoidea are the most heavily mineralized, and the individual skeletal elements are single crystals with few exceptions [(1) and the references therein]. The Echinoidea proteins implicated in mineral formation have great similarities with those involved in mammalian mineralization, despite use of calcium carbonate by the former and calcium phosphate (hydroxyapatite) by the latter, and this motivates studies of echinoids from a biomineralization perspective. Echinoid skeletal element morphologies allow identification down to the species level, and these ossicles are often the only part of the animal fossilized.

Correspondence: Stuart R. Stock, Department of Molecular Pharmacology and Biological Chemistry, Feinberg School of Medicine, Northwestern University, 303 E. Chicago Ave., Chicago, IL 60611, USA. s-stock@northwestern.edu.

*Present address: Diamond Light Source, Harwell Campus, Didcot, Oxon, OX11 0DE, UK.

†Present address: Office of Basic Energy Sciences, SC-22.3/Germantown Building, U.S. Department of Energy, 1000 Independence Avenue, SW, Washington, D.C. 20585, USA.

Declaration of interest

The authors have no financial interest in any of the results or interpretations reported in this article.

The jaw of the sea urchin (termed Aristotle's lantern) consists of five pyramids, and each of these contains a single continuously growing tooth which contains all stages of tooth formation. Each tooth is a single crystal, or more precisely, a bi-crystal with a small disorientation of 5–10° (2–5) consistent with echinoderms being members of Bilateria. Across the orders of the regular sea urchins, tooth cross-sections range from grooved, wedge-shaped, prism-shaped to T-shaped.

The calcite of regular sea urchin teeth forms in two stages. The first-forming consists of plates (primary, secondary and, in some taxa, carinar process), the lamellae-needle complex and needle-prisms. All form in spaces between cellular syncytia. The second stage fills the space between adjacent first stage mineral elements (2,6–10). Like their names imply, the first stage mineral elements develop initially in the plumula (most aboral portion of the tooth) and the second stage mineral (in the form of columns or disks) subsequently links the plates and prisms together. All of the calcite in the sea urchin tooth contains significant Mg substituted for Ca, i.e. $\text{Ca}_{1-x}\text{Mg}_x\text{CO}_3$. The columnar interconnections between primary plates are a very primitive character dating back at least to the Carboniferous (>300 Ma) (11).

Earlier, synchrotron microbeam diffraction measured the range of calcite compositions in *Lytechinus variegatus* (Lamarck, 1816) (Echinoida, Toxopneustidae) teeth (3,5), and composition agreed with that reported earlier for ground teeth of this species (12). By studying intact or cross-sectioned teeth, the diffraction studies also showed that the first and second stage mineral in *L. variegatus* had the same crystallographic orientation, despite the origin of the first stage mineral elements in independent syncytial spaces (3,5). Given that many investigators have shown the columns of very high Mg calcite ($0.3 < x < 0.4$) are found only in contact with the first stage plates and prisms of high Mg calcite ($0.1 < x < 0.2$), (3,5) argued that the observed co-registration of high and very high Mg calcites may very well be epitaxy. Diffraction studies on teeth of *Paracentrotus lividus* (Lamarck, 1816) (Echinoida, Parechinidae) revealed a similar crystallographic relationship between first and second stage phase (4); and this led the authors of this study to present diffraction data evaluating whether this co-registration/epitaxy occurs in sea urchin teeth from four families of Echinoida and from orders Temnopleuroidea, Arbacioida and Cidaroida. In the process of investigating the co-registration/epitaxy of first and second stage mineral, the ranges of magnesian calcite compositions in these teeth were also determined.

Second stage mineral cements the first stage mineral elements together, an essential final step in producing a functional tooth. How the same cellular syncytia switch from producing a lower Mg calcite (first stage mineral) to a higher Mg calcite (second stage mineral) has not yet been established, and this remains an important biomineralization question. A related unanswered question and the subject of this article is whether first and second stage mineral co-orientation/epitaxy observed in two families of Echinoida (3–5) is present in other orders of regular sea urchins. Observation of common co-orientation/epitaxy relationships across the disparate orders reported here suggests that this crystallographic relationship is a synapomorphy of the regular sea urchins and that observations on second stage mineralization of one species apply to species of orders as evolutionarily remote as Echinoida and Cidaroida.

Materials and methods

Table 1 lists the teeth examined. All teeth were dried, and some specimens were cross-sections [prepared for microbeam diffraction mapping, e.g. (3)] and others were intact teeth. Despite the x-ray beams being oriented differently in the two cases (i.e. relative to the tooth's crystal axes), the crystallographic relationship of the first- and second stage mineral could still be examined. Examining intact teeth allows subsequent microCT or other studies covering the contiguous volume. The following whole teeth were studied: *Eucidaris tribuloides* (Lamarck, 1816), *Tetrapygyus niger* (Molina, 1782), *Mespilia globulus* (Linnaeus, 1758) and *L. variegatus*. Cross-sections of the following teeth were prepared by casting them in LR-White (Electron Microscopy Sciences, Hatfield, PA) and by sectioning them transversely to ~0.65 mm thicknesses using an Isomet 1000 wafering saw (Buehler, Lake Bluff, IL): *Heterocentrotus trigonarius* (Lamarck, 1816), *Mesocentrotus franciscanus* (Agassiz, 1863), *P. lividus* and *L. variegatus*. All of the teeth except *E. tribuloides* are "T"-shaped, i.e. stirodents or camarodents, whereas *E. tribuloides* is grooved. Transverse cross-sectional microCT images of teeth of these or closely related species have been published elsewhere (e.g. (13)), and microCT slices of teeth of the above species are shown in Supplementary Figure 3 for clarification.

Transmission diffraction patterns were recorded at stations 1-BM and 1-ID of the Advanced Photon Source. At each station, the image plate detector (Mar345, MAR Research, www.marresearch.com) was positioned perpendicular to incident beam (Supplementary Figure 1). In the patterns recorded at 1-BM (20.019 keV photon energy), the incident beam position was offset significantly from the detector's center; at 1-ID (80.7 keV photons), the incident beam position was near the center. These two energies are typical for the two stations and were required for other samples studied during the same beam time; comparisons made through crystal *d*-spacings are not affected by use of the two energies. Supplementary Table 1 gives other experimental details for the transmission diffraction experiments.

Diffraction patterns from the transversely sectioned teeth were recorded at 1-ID, and the cross-sections were from the most adoral section of the tooth that showed the complete tooth cross-section (i.e. as close as possible to the incisal end). The sections were positioned perpendicular to the incident beam, and the teeth were translated across the beam in order to collect patterns from different regions of the tooth (Supplementary Figure 1a).

Diffraction patterns of the whole teeth were recorded at 1-BM, and the tooth inside a thin plastic tube (internal diameter ~4 mm) was positioned vertically, that is, with its axis parallel to the diffractometer's rotation axis (*Y*-axis). Sea urchin teeth curve significantly, and the teeth were positioned with this curvature in the plane defined by the incident beam (*Z*-axis) and the vertical axis (Supplementary Figure 1b). This orientation minimized the number of *X* translations required to cover the tooth width but had the consequence of producing sample-detector separations estimated to vary by ± 2 mm over the length of the tooth. Diffraction patterns were collected across the tooth width and along its length from the middle of the tooth shaft to its incisal end, positions where the volume of second stage mineral is significant. Because the Ewald sphere diameter is rather small at 20 keV

compared to that at 80 keV, the teeth studied at 1-BM were oscillated slightly about the Y -axis while each pattern was collected (Supplementary Table 1); this brought more $hk.l$ to the Bragg condition.¹

Diffraction patterns from a thin standard of CeO_2 powder were recorded and used to refine the sample-detector distance and to account for detector tilts (3). The 2D diffraction patterns were inspected for double or triple diffraction spots (i.e. diffraction peaks extending over the same azimuthal range on the detector and with slightly different radial positions). The coordinates X_{det} , Y_{det} of the center of each diffraction spot were measured, and the radial separation of the spot and the incident beam position was converted into diffraction angle 2θ and, via Bragg's law, into d -spacing. Indices $hk.l$ for each group of diffraction spots were assigned using the ICDD Powder Diffraction File cards 86-2335 ($x = 0.064$) and 86-2336 ($x = 0.129$). Conversion of d -spacing to composition x of $\text{Ca}_{1-x}\text{Mg}_x\text{CaO}_3$ (calcite) was done by constructing linear calibration curves for the respective reflections using the above cards and card 36-426 ($x = 0.5$). This linear calibration curve agrees with (14). Card 86-2343 ($x = 0$) was not used because the Mg compositions within the sea urchin teeth are greater than 0.06 and the d -spacings for calcite with $x = 0$ are non-linear compared to those of the other three compositions.

The uncertainties in sample-detector separation due to curvature of the whole teeth (data from 1-BM, ± 2 mm) are large enough to shift the absolute values of x . For $x = 0.11$, an actual separation 2 mm larger than the calibrated distance produces $x = 0.07$ and 2 mm smaller $x = 0.14$. The values of x for the doublet or triplet spots are equally affected, however, and comparisons of the range of compositions x for the doublet/triplet of each $hk.l$ are valid for all of the sea urchin teeth and are reported below instead of individual x .

Results

Supplementary Figure 2 shows complete diffraction patterns of *M. globulus* and *M. fransiscanus* recorded at stations 2-BM and 1-ID, respectively. For clarification, Supplementary Figure 3 shows microCT slices of the transverse cross-section of the various teeth. Except for differences in orientation, these patterns are typical of those recorded at the two stations. In each pattern, an arrowhead indicates a diffraction spot showing particularly visible radial doublets. Some of the diffraction spots are very weak and show only one d -spacing; these diffraction planes were far from the exact Bragg condition, and only the most intense component of the different calcite constituents (first stage mineral making up the majority of the tooth volume) produced measurable diffraction intensity. This is consistent with the differences in intensity for the individual doublet/triplet peaks in Figure 1.

Figure 1 shows enlargements of “single” diffraction spots of each species studied, with the left column of images (a–d) recorded with 80 keV X-rays and the right column (e–h) with 20 keV. Note that there are two *L. variegatus* specimens, one studied at each energy. Two or more distinct compositions are seen for each specimen (i.e. slightly different radial positions

¹Note the use of Miller-Bravais indices for this hexagonal crystal system. The period denotes the third axis in the $hkil$ indexing system which need not be written, given $i = -(h + k)$, but emphasizes the hexagonal crystal system.

corresponding to small differences in d for the same $hk.l$), and black and white arrows label the outer- and inner-most reflections within each enlargement. The azimuthal displacement between radial doublets or triplets never exceeds more than the few degrees produced by non-optimum alignment of the diffraction planes relative to the incident beam.

The peaks within each $hk.l$ reflection take two types of shapes. The first are groups of individual sharp equiaxed spots (i.e. roughly equal radial and azimuthal dimensions) lying along the diffraction rings. The second are smooth arcs extending over $\sim 5^\circ$ azimuthally. The equiaxed spots appear at the smaller 2θ (larger d and smaller x) within the doublet/triplets whereas the arcs are seen both at larger and smaller 2θ . In some of diffraction spots, the bi-crystal nature of the tooth is revealed (e.g. Figure 1c and g): pairs of arc-like diffraction spots or of groups of small spots with the same radius but separated by $5\text{--}10^\circ$ azimuthal rotation.

Table 1 gives values for x for the different species. The teeth had various orientations relative to the beam, and different $hk.l$ are oriented for diffraction and used to determine x . The values of x consistently approach 0.2 or greater. The largest x observed is 0.29 for the 02.4 reflection of *L. variegatus*, but the two 10.4 reflections in the same diffraction pattern have values of 0.22 and 0.23. The smallest x observed is 0.15 for the 10.4 reflection of *E. tribuloides*; the Ewald sphere simultaneously intersects the second order reciprocal lattice point for the same diffraction planes (i.e. 20.8) and reveals a somewhat larger $x = 0.23$. Values of x from the literature include 0.35 (5), 0.33 (12) and 0.19 (3) for *L. variegatus*; 0.23 (8) for *Sphaerechinus granularis* (a member of the same family as *L. variegatus*) and 0.30 (4) for *P. lividus*.

Discussion

The diffraction patterns (Figure 1) show the high Mg calcite and very high Mg calcite phases have the same crystallographic orientations in the teeth from four families of order Echinoida and from orders Temnopleuroidea, Arbacioidea and Cidaroida. Among the extant regular sea urchins, the cidaroid sea urchins are, in evolutionary terms, the farthest from Echinoida. This shows that co-orientation of the phases is a characteristic of the regular sea urchins and implies that teeth from the orders intermediate between Arbacioidea and Cidaroida (i.e. Diadematoidea, Echinothurioida) will, when examined by diffraction, likewise have the same crystallographic relationship between high and very high Mg calcites. Optical microscopy with polarized light (e.g. (2,8)) agrees with the diffraction results except for the “accessory” calcite of echinothuroid teeth (which have an orientation differing from the rest of the teeth (15)) and for structures in clypeasteroid teeth (16). The observation of solid columns linking plates in fossils from the late Carboniferous (11) suggests the co-orientation of first and second stage mineral may, in fact, be a very primitive character, but one that will be exceedingly difficult to establish given recrystallization within the fossils.

The range of compositions x characteristic of these diffraction patterns cluster around $0.2 < x < 0.25$ and are no smaller than 0.15 and no larger than 0.29. For both of the extreme values, however, other reflections in the same diffraction pattern yield x closer to the characteristic range. Comparable or somewhat larger x have been reported in the literature.

The present values of x in Table 1, however, represent a minimum composition range: larger or smaller x may be present in volume fractions too low to be detected or in regions not have been sampled by the x-ray beam. The values of x also reflect only the Mg that is present in calcite and not Mg that is present in organic material or any amorphous mineral phases.

Two diffraction peak morphologies are observed within each $hk.l$, smooth arcs and groups of sharp, equiaxed peaks, consistent with observations of transversely sectioned *L. variegatus* teeth (3). The discrete substructure, seen only from the first stage calcite, i.e. with lowest x , merges into a smooth arc when more material is present. All of the sea urchin teeth examined curve along their lengths, and the azimuthal “splitting” into “sub-spots” reflects small rotations of the first stage structural elements within the volume sampled. Small increments of rotation from identical plate to identical plate produce a macroscopically curved structure, and this is one type of structural gradient. The authors speculate that each plate at the same stage of development has the same orientation, and the small rotations (between adjacent plates) occur due to the sequential advance of each plate within the highly curved plumula (e.g. Figure 1 of (5)). Macroscopically, the amount of tooth curvature is fixed when the second stage mineral connects all of the crystalline material. Such patterns of small misorientations within single grains are common in metal single crystals.

The data clearly show the first and second stage mineral have the same crystallographic orientations, and the tooth might be regarded as compositionally modulated crystal. One question is whether the second stage grows epitaxially on the pre-existing first stage mineral. Numerous studies have shown that very high Mg calcite columns nucleate at many different locations between adjacent first stage plates or prisms and that the two phases are in contact or, at a minimum, are separated by a very thin layer of organic material (10). If the two crystal phases are in direct contact, the case would very strong for an epitaxial relationship, albeit with an incoherent interface. If the two phases were separated completely by a thin organic phase, one might regard this as a sort of buffer layer, like those used in molecular beam epitaxy to accommodate strains due to mismatched lattice parameters.

Atomic resolution transmission electron microscopy (TEM) is required to determine exact nature of the interface, specifically, continuity across the interface as opposed to slightly separated but co-oriented crystals. Sea urchin calcite is a difficult material to study with conventional TEM as it is unstable under electron irradiation, but cryoTEM or atomic force microscopy may sidestep this limitation and provide direct evidence for or against epitaxy of the high and very high Mg calcites in the sea urchin tooth.

The reported common co-orientation/epitaxy relationships across the disparate orders examined suggests that this crystallographic relationship is a synapomorphy of the regular sea urchins. Synapomorphy implies, in turn, that observations on second stage mineralization of one species apply to species of orders as evolutionarily remote as Echinoida and Cidaroida. It is important to emphasize that the second stage mineral is the essential final step in production of functional teeth in the sea urchin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Diffraction spots enlarged and in the same orientation as on the detector. The same color bar is used for each image, all are log scaled except (b) which is linear. For each species, the following information is given: tooth status (cross-section or whole tooth), hkl reflection shown, vertical $\times$ horizontal image dimensions in detector pixels and the maximum and minimum intensities (in cts) within each enlargement indicated as [min, max]. (a) *H. trigonarius*, cross-section, 10.4, 100×100 , [10^2 , 7×10^4]. (b) *M. fransiscanus*, cross-section, 10.4, $100 \times 100 \mu\text{m}^2$, [0, 4.3×10^4]. (c) *P. lividus*, cross-section, 20.8, $100 \times 100 \mu\text{m}^2$, [20, 3×10^3]. (d) *L. variegatus*, cross-section, 10.4, $100 \times 100 \mu\text{m}^2$, [50, 1.2×10^4]. (e) *E. tribuloides*, whole tooth, 20.8, $100 \times 150 \mu\text{m}^2$, [10^2 , 2.2×10^3]. (f) *T. niger*, whole tooth, 11.3, $200 \times 150 \mu\text{m}^2$, [10^2 , 7×10^5]. (g) *M. globulus*, whole tooth, 11.3, $150 \times 100 \mu\text{m}^2$, [2.5×10^2 , 5×10^5]. (h) *L. variegatus*, cross-section, 20.8, $200 \times 150 \mu\text{m}^2$, [10, 1.2×10^3]. White or black arrows indicate the diffraction spots with maximum and minimum Mg compositions. The white disk indicates the portion of each enlarged diffraction spot closest to the incident beam.

Sea urchin teeth studied by synchrotron transmission X-ray diffraction and whether teeth were prepared (i.e. whole teeth “w” versus cross-sections “xs”), X-ray energy, $hk.l$ indices, and composition difference x between first and second stage calcite.

Table 1

Species	Order family	w/xs	Location; date; collector	Energy (keV)	$hk.l$	x
<i>Eucidaris tribuloides</i>	Cidaroida Cidaridae	w	Panacea, FL, USA; 2003; Gulf Specimen Marine Lab.	20.1	10.4 20.8	0.15 0.23
<i>Tetrapygyus niger</i>	Arbacioida Arbaciidae	w	Chile; 2003; J. Arias.	20.1	11.3 11.3 02.4 12.2	0.19 0.20 0.20 0.19
<i>Mespilia globulus</i>	Tennopleuroida Tennopleuridae;	w	Enewetak Atoll, Marshall Is, 1982; T. Ebert.	20.1	11.0 11.3 20.2	0.22 0.24 0.22
<i>Lytechinus variegatus</i>	Echinoidea Toxopneustidae	w, xs	Panacea, FL, USA; 2003; Gulf Specimen Marine Lab.	20.1	10.4 10.4 02.4	0.22 0.23 0.29
<i>Heterocentrotus trigonarius</i>	Echinoidea Echinometridae	xs	Enewetak Atoll, Marshall Is, 1978; T. Ebert.	80.7	10.4	0.22
<i>Mesocentrotus franciscanus</i>	Echinoidea Strongylocentrotidae	xs	San Nicolas Is, CA, 8–10 m; 1991; M. Russel, J. Estes.	80.7	10.4	0.18
<i>Paracentrotus lividus</i>	Echinoidea Parechinidae	xs	Europe; 2003; S. Weiner.	80.7	10.4 20.8	0.19 0.21