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Calcium sulfate hemihydrate in statoliths of deep-sea medusae

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We report on the examination of the statoliths of the coronate medusa *Periphylla periphylla*. The statoliths are single crystals of calcium sulfate hemihydrate of about 60 μm length and 15 μm diameter, as identified by powder and single-crystal diffractometry at the synchrotron. This is most surprising, as this phase (gypsum plaster, the mineral bassanite) can not usually be precipitated from aqueous solutions and easily takes up water to yield the dihydrate of calcium sulfate (hardening reaction of gypsum). As it is sensible to use the considerably more dense hemihydrate instead of the dihydrate as a gravity sensor (32% higher density after buoyancy correction), it can be concluded that this biological system actively induces the crystallisation of this phase far away from chemical equilibrium. As a biomineral, calcium sulfate hemihydrate has not been found in living organisms before. The results are complemented by a discussion of histology and evolutionary aspects.

Introduction

Few data on the mineral composition of the gravity sensors of medusae, the so-called statoliths, are known. The only reports comprise the presence of calcium sulfate dihydrate (gypsum) in the biological classes Semaeostomeae (Scyphozoa)[1–3] and Cubozoa[4] and calcium magnesium phosphate in Hydrozoa.[4,5] In order to clarify the evolutionary relationships of the classes Scyphozoa, Cubozoa and Hydrozoa within the phylum Cnidaria (Coelenterates, comprising sea anemones, jellyfish, hydras, sea fans and corals)[6] we have studied samples of the deep sea medusa *Periphylla periphylla* (Scyphozoa, Coronatae) that lives in depths between 200 and 2000 m.[7] Their complex marginal sense organ (rhopalium) is responsible for photoreception, equilibrium reception and sensory responses to other stimuli such as touch, chemicals, pressure and temperature (Fig. 1). The gravity sensors inside the rhopalia are the statocysts containing crystalline statoliths.

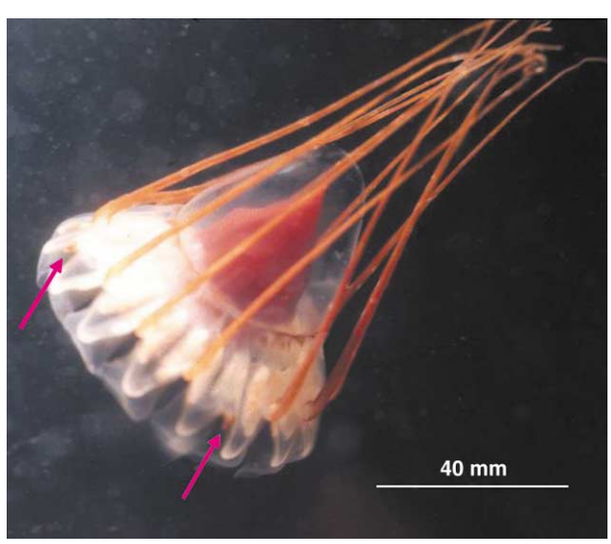

Fig. 1 The medusa *Periphylla periphylla* with positions of statocysts indicated (arrows).

Results and discussion

TEM observations of demineralised material show specialised entodermal cells which wrap the vacuoles of the crystals. Furthermore the vacuoles themselves are wrapped by a thin cellular layer of about 80 nm diameter. These cells are based on the mesogloea (the gelatinous non-cellular layer between the endoderm and the ectoderm in the body wall) but some reach the centre of the statocyst. They are filled up with 0.5(1) μm vesicles with an as yet unknown function, but we believe that these vesicles contain the concentrated solutions which are necessary for crystal formation (similar to matrix vesicles in bone formation). These vesicles appear to leave the surface of the cells to contact the wrapping cellular layer, followed by unloading of the pre-formed mineral (Fig. 2).

The investigations by SEM show well-shaped trigonal crystals of 60 μm length with a diameter of 15 to 20 μm (Fig. 3). The surface of the crystals is covered by a layer of spherical particles of 50 nm diameter. Assuming that the crystals consist of calcium sulfate hemihydrate (see below), the crystal faces were indexed as {3 0 2} (headfaces) and {1 0 0} (sidefaces), respectively. The elemental analyses performed with EDX-spectroscopy showed mainly Ca, S and O, besides traces of Na and K from the surrounding tissue (or possibly from incorporated ions) and Al from the sample holder, indicating the presence of a calcium sulfate mineral.

The crystallographic phase was identified by synchrotron X-ray powder diffractometry. The very small amount of substance gave diffractograms of only moderate quality that nevertheless permitted a clear identification of the phase. To our surprise we found that the statoliths consist of calcium sulfate hemihydrate ($CaSO_4 \cdot 0.5H_2O$) and *not* of the dihydrate ($CaSO_4 \cdot 2H_2O$), *i.e.* gypsum (Fig. 4), as we had expected. The structural parameters of the hexagonal unit cell were refined to $a = 6.9686$ Å and $c = 6.3600$ Å (from nine reflections), in good agreement with the literature values of 6.937 and 6.345 Å.[8]

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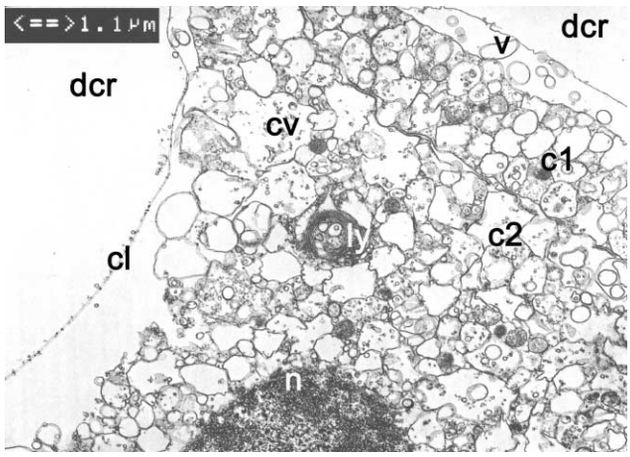

Fig. 2 Transmission electron micrograph of the statolith cells of *Periphylla periphylla* (note that the statoliths were dissolved during preparation). c1, c2 cells; cl cellular layer around the statoliths; cv cellular vesicle; dcr dissolved crystal (site of the statolith); ly lysosome; n nucleus; v vesicle.

The basic structural feature of all calcium sulfate subhydrates are chains of alternating Ca^{2+}-ions and SO_4^{2-}-polyhedra parallel to [001]. These chains build up structural channels which are able to accommodate different amounts of water molecules as a guest phase. Therefore, a range of non-stoichiometric calcium sulfate hydrates (from zero to almost one water molecule per calcium) is known.[8] It is very difficult to distinguish between these phases by powder diffraction. Consequently, the crystallographic nature of the statoliths was explored further by single-crystal X-ray structure analysis at the synchrotron. Despite the moderate data/parameter ratio, Patterson and difference Fourier methods clearly revealed oxygen atoms (O3) of water molecules within the structural channels along the *c*-axis. Significant residual electron densities and elongated O3 thermal ellipsoids (along [001]) indicate a substantial degree of disorder of the water molecules.

The fact that the statoliths consist of calcium sulfate hemihydrate $CaSO_4 \cdot 0.5H_2O$ is most astonishing as this phase cannot be precipitated from water under normal conditions. In fact, this compound is always metastable compared to $CaSO_4 \cdot 2H_2O$ ("gypsum") and $CaSO_4$ ("anhydrite"). The equilibrium temperature for the dehydration of dihydrate to anhydrite is around 38–42 °C, and the equilibrium temperature for the dehydration of dihydrate to hemihydrate is around 97–107 °C. The presence of very high concentrations of inorganic ions can induce the precipitation of hemihydrate at 17 °C (saturated $CaCl_2$ solution: *ca.* 740 g l^{-1}) or 11 °C (saturated $MgSO_4$ solution: *ca.* 260 g l^{-1}), respectively. Under the given environmental conditions for *Periphylla periphylla* (water at about 6 °C, salt concentration about 35 g l^{-1}, mostly NaCl), any calcium sulfate hemihydrate should take up water immediately to form the dihydrate, in analogy to the technical process of gypsum hardening.[9]

If we accept the presence of calcium sulfate hemihydrate in the statoliths, two important conclusions can be drawn. First of all, the formation of hemihydrate must occur in well secluded compartments (probably the vesicles found in TEM) under the nucleating influence of specialised biomolecules. Such mechanisms have been identified during the biomineralization of molluscs and successfully simulated *in vitro*.[10–12] Second, the crystals must be permanently protected towards the aqueous environment in order to prevent hydration. This is probably accomplished by a thin layer of organic matter (Figs. 2 and 3c) covering the statoliths. This view is supported by the observation that upon storage of the explanted statocysts in pure water, this membrane is destroyed and the statoliths dissolve. The ion product of calcium and sulfate in seawater is about 1.5×10^{-5} $mol^2\ l^{-2}$ (Ca^{2+}: 1.4 mmol l^{-1} = 0.08 g l^{-1}, SO_4^{2-}: 11 mmol l^{-1} = 1.06 g l^{-1}), well below the solubility product of calcium sulfate

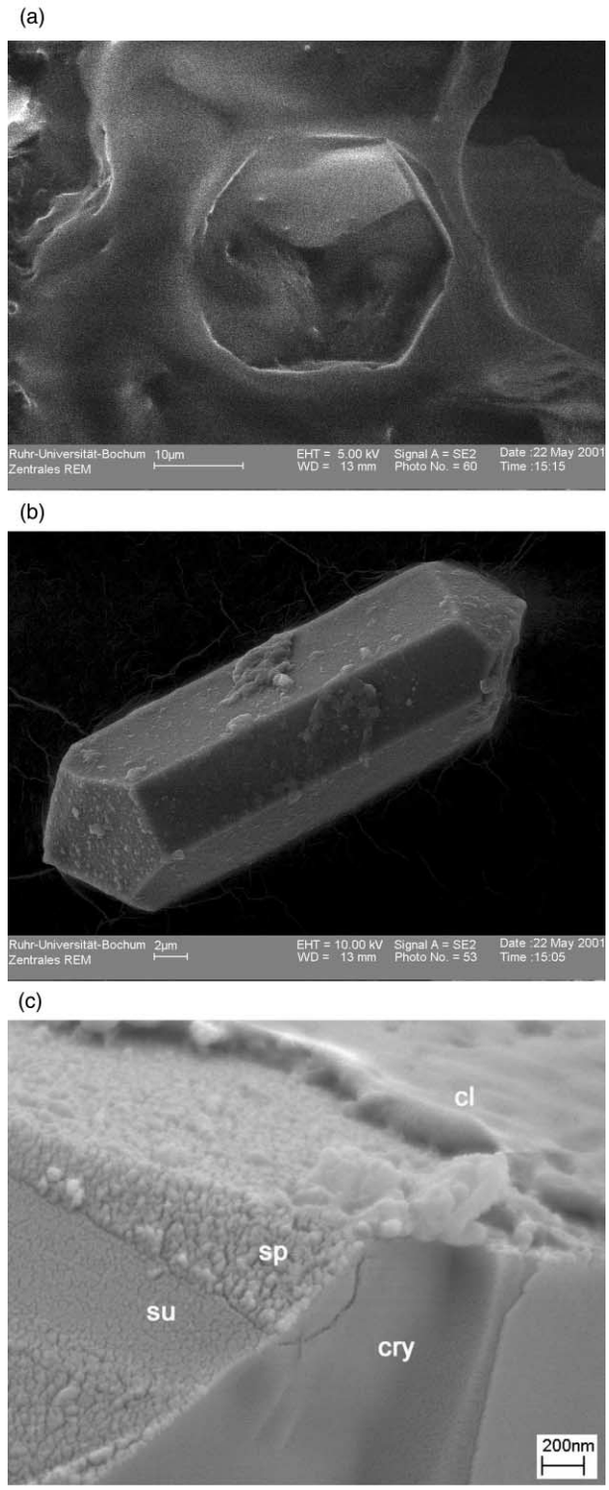

Fig. 3 Scanning electron micrographs of the statoliths of *Periphylla periphylla* with a view on {3 0 2} faces (a), on the {1 0 0} faces (b), and on a fracture surface, showing the organic coating on the surface (c). cl cellular layer; cry crystal (statolith); su surface of the statolith; sp spherical particles coating the crystal.

at 6 °C (*ca.* 4.7×10^{-3} $mol^2\ l^{-2}$), therefore dissolution of unprotected statoliths would occur even in the living organism.

From a biological point of view, it makes sense to use calcium sulfate hemihydrate instead of calcium sulfate dihydrate as a gravity sensor. The density of the hemihydrate is higher (2.73 g cm^{-3}) than that of the dihydrate (2.31 g cm^{-3}). If we eliminate the buoyancy by subtracting the density of water, the density ratio becomes even more favourable (1.73 : 1.31). In general, $CaSO_4$ and its hydrates are very rare biominerals.[6,13] Besides statoliths in medusae there were early reports about gypsum

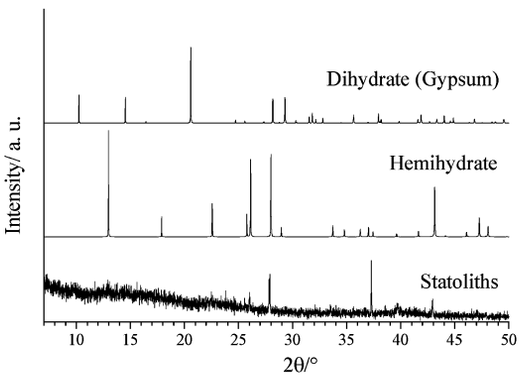

Fig. 4 X-Ray powder diffractogram of intact statocysts (containing the statoliths; strongly preferred orientation) and patterns simulated for calcium sulfate dihydrate (gypsum) and hemihydrate (bassanite).

crystals in green algae of the family Desmidiaceae especially in the genus *Closterium*.[14] Later, these crystals were identified as $BaSO_4$ with traces of $SrSO_4$.[15] In some species of the same family needles of calcium sulfate were found that are also supposed to serve as statoliths.[16]

The presence of calcium sulfate in statoliths of *Periphylla periphylla* (Scyphozoa, Coronatae) gives important hints on the evolution of medusae. Fossils confirm that the origin of the Cnidaria lies in the Precambrian (*i.e.* older than 570 million years).[17] All found medusae of those early times are classified within the Scyphozoa. The fact that they are using calcium sulfate for their statoliths up to now demonstrates the close relationship between the classes Scyphozoa and Cubozoa which is confirmed by other characteritics shared by both groups, *e.g.* gastric filaments and rhopalia as marginal sense organs originating from transformed polyp tentacles. Species of the third metagenetic class of Cnidaria, the Hydrozoa, use calcium/magnesium phosphate in their statocysts.[4,5] Our results strongly support the hypothesis that medusae have developed twice within the Cnidaria: on the one hand in the Scyphozoa and Cubozoa and on the other hand in the Hydrozoa.

One final word towards a potential technical significance: If it would be possible to precipitate calcium sulfate hemihydrate from aqueous solutions triggered by such biomolecules, it would save us the procedure of thermally transforming about 93 million tons of dihydrate per year into hemihydrate for construction purposes.

Experimental

Samples of *Periphylla periphylla* were taken by MIK (Modified Isaak Kidd, diameter 2 m, 500 μm mesh) and multinet from Norwegian fjords (Research Vessel *Haakon Mosby*, depth between 200 and 1200 m). After preliminary examination of the structure of the rhopalia from the living material by microscopy, the marginal sense organs were kept in 80% ethanol during the transport for about 3 months. In order to exclude the possibility that initially present dihydrate had transformed into hemihydrate during storage, we subjected a sample of ground dihydrate to the same procedure for one week, followed by X-ray diffraction analysis. There was no transformation of dihydrate into hemihydrate, but on the contrary, traces of hemihydrate in the sample had converted into dihydrate. The presence of hemihydrate in the statoliths therefore cannot be an artefact due to sample preparation. Further support comes from the observation of well-shaped single crystals of hemihydrate that are very unlikely to result from a wet-chemical conversion (they also have the same shape in freshly dissected animals). For scanning electron microscopy (SEM) and single-crystal diffraction, the statoliths were isolated from the surrounding tissue and dried. The samples were sputtered with gold for SEM analysis. For histological examinations and transmission electron microscopy (TEM) the material was fixed in glutaraldehyde/seawater which dissolved the crystals. For synchrotron powder diffractometry, the intact statocysts were used (*i.e.* with the crystals inside the tissue; not ground). High-resolution X-ray powder diffractometry was carried out in transmission geometry at beamline B2 at HASYLAB/DESY, Hamburg, Germany, with $\lambda = 1.35724(2)$ Å. Single-crystal diffraction was carried out at beamline F1 at HASYLAB/DESY with a microcrystal of $20 \times 18 \times 55$ μm^3 ($\lambda = 0.6229$ Å, CCD-detector, exposure time 15 s/frame, stepwidth $\Delta\omega = 0.2°$). Weak reflections were observed up to a resolution of 0.57 Å and unambiguously indexed in the space group $P3_121$ with $a = 6.984(1)$ Å and $c = 6.378(1)$ Å. 565 reflections with $F^2 > 4\sigma(F^2)$ (984 unique data, $R_{int} = 0.168$) were used for structural analysis. The final refinement values were $R_1 = 0.1591$ and $wR_2 = 0.5135$.

Acknowledgements

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