Journal of Structural Biology 189 (2015) 28–36

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Layered growth of crayfish gastrolith: About the stability of amorphous calcium carbonate and role of additives

Wouter J.E.M. Habraken [a,*], Admir Masic [a], Luca Bertinetti [a], Ali Al-Sawalmih [b], Lilah Glazer [c], Shmuel Bentov [c], Peter Fratzl [a], Amir Sagi [d], Barbara Aichmayer [a], Amir Berman [e]

[a] Max Planck Institute of Colloids and Interfaces, Department of Biomaterials, 14424 Potsdam, Germany
[b] Marine Science Station (MSS), The University of Jordan/Aqaba Campus, 77110 Aqaba, Jordan
[c] Life Sciences Dept., Ben-Gurion University of the Negev, 84105 Beer-Sheva, Israel
[d] Life Sciences Dept. and the National Institute for Biotechnology in the Negev, Ben-Gurion University, 84105 Beer-Sheva, Israel
[e] Biotechnology Engineering Dept., Ben-Gurion University of the Negev, 84105 Beer Sheva, Israel

ARTICLE INFO

Article history:
Received 12 September 2014
Received in revised form 17 November 2014
Accepted 18 November 2014
Available online 26 November 2014

Keywords:
Amorphous calcium carbonate
Crayfish
Gastrolith
Hierarchical organization
Stabilization

ABSTRACT

Previous studies on pre-molt gastroliths have shown a typical onion-like morphology of layers of amorphous mineral (mostly calcium carbonate) and chitin, resulting from the continuous deposition and densification of amorphous mineral spheres on a chitin-matrix during time. To investigate the consequences of this layered growth on the local structure and composition of the gastrolith, we performed spatially-resolved Raman, X-ray and SEM–EDS analysis on complete pre-molt gastrolith cross-sections. Results show that especially the abundance of inorganic phosphate, phosphoenolpyruvate (PEP)/citrate and proteins is not uniform throughout the organ but changes from layer to layer. Based on these results we can conclude that ACC stabilization in the gastrolith takes place by more than one compound and not by only one of these additives.

1. Introduction

Only recently it was discovered that Nature uses amorphous materials as precursor phases preceding the formation of elaborate crystalline structures and tissues, like the spicules of sea-urchins (Beniash et al., 1997; Politi et al., 2004), zebrafish bone (Mahamid et al., 2011) and in many more cases (Addadi et al., 2003). In those examples the formation of an eventual crystalline material (i.e. calcite, aragonite, or apatite) proceeds via an amorphous precursor state that under *in vitro* conditions is often only present as a kinetically stabilized intermediate (Sawada, 1997).

In contrast to pure synthetic amorphous calcium carbonate or phosphate, in biological samples, the amorphous phase can also be much more stable against thermodynamic pressure, forming functional end-products like the crayfish exoskeleton (Bentov et al., 2012) without transforming into a crystalline polymorph. This phenomenon is speculated to result from the presence of molecular agents that stabilize the amorphous phase and delay crystal formation. From studies on skeletons of marine organisms a wide scale of such stabilizing agents is proposed. Examples are highly charged and/or phosphorylated proteins, small organic molecules, foreign ions (especially Mg^{2+} (Politi et al., 2010)) and specialized macromolecules (Aizenberg et al., 1996). Studies on synthetically grown calcium carbonate or calcium phosphate under physiological conditions have shown that highly charged polymers like poly(aspartic acid) are able to stabilize a so-called Polymer-Induced Liquid Precursor (PILP) phase (Olszta et al., 2007). PILP represents a highly hydrated non-crystalline mineral phase, which is believed to resemble amorphous precursors in many biological systems. Additionally, the influence of (small) organic molecules as well as foreign ions on *in vitro* calcium carbonate/calcium phosphate growth has been studied since the 1970's. These studies show that it is possible to delay the reaction kinetics (and thereby increase the stability of the amorphous phase) quite radically by only introducing a small amount of mostly acidic agents or polyvalent ions (f.e. pyrophosphate, casein, Mg^{2+}, SO_4^{2-}) (Termine et al., 1970; Ihli et al., 2013). Non-acidic agents or monovalent ions (f.e. Na^+, gelatin), in general, leave the reaction unaffected.

In this study we investigated the stable amorphous calcium carbonate (ACC) in the crayfish temporary mineral storage, the gastrolith. Crayfish need a large amount of mineral during the molting cycle. Therefore, some species of fresh-water crayfish have a specialized storage-the gastrolith, first described by Huxley

* Corresponding author.
E-mail address: wouter.habraken@mpikg.mpg.de (W.J.E.M. Habraken).

http://dx.doi.org/10.1016/j.jsb.2014.11.003
1047-8477/

(1880). Gastroliths are located between the endocuticle and epidermis of the gastrolith disks (Travis, 1963), organs dedicated to the production of the gastrolith, which are situated on both sides of the stomach. The growth of the gastroliths involves first the deposition of a poorly ordered lamellar α-chitin matrix, with orthogonally directed shorter chitin fibers. Accordingly, the amorphous mineral attaches on this matrix as nanometer-sized spheres (Travis, 1963). In this process, both the chitin matrix and the mineral particles are secreted by the epidermal cells of the gastrolith disk. As the gastrolith matures, new mineral is deposited on the external layer, while the earlier deposited mineral densifies into macroscopic columnar structures, directed parallel to the growth direction. In time, the composite of the lamellar chitin and the mineral forms an ordered structure of concentric layers (Travis, 1963), which is finally resorbed through collapse of the tissue into the crayfish stomach during ecdysis (Shechter et al., 2008a).

It is postulated that certain proteins found in the crayfish gastroliths take part in the mineralization of the chitin scaffold by playing different roles in modulating mineralization (i.e. stabilizing ACC) and attaching the chitin to the mineral. These proteins include the gastrolith matrix protein (GAMP) (Takagi et al., 2000), gastrolith protein 65 + 75 (GAP65 + GAP75) (Shechter et al., 2008b; Glazer and Sagi, 2012), CqCDA1 (Yudkovski et al., 2010) and gastrolith protein 10 (GAP10) (Glazer et al., 2010)). Conversely, recent ss-NMR studies (Sato et al., 2011; Akiva-Tal et al., 2011) indicate that small organic molecules like phosphoenolpyruvate (PEP) and citrate but most of all inorganic phosphate (Akiva-Tal et al., 2011), present in gastroliths up to an average of maximally 18 wt%, are the most likely candidates to stabilize ACC in the gastrolith. Note that unlike Mg^{2+}, inorganic phosphate is not a substitutional impurity in any crystalline calcium carbonate. Furthermore, the chemical environment of the phosphate elucidated in ss-NMR (Akiva-Tal et al., 2011) indicates that it is well dispersed inside the ACC structure. These observations, however, do not rule out that a combination of several factors (proteins, inorganic phosphate, small organic molecules) could act cooperatively to enhance the stability of gastrolithic ACC. Additionally, taking into account the prospected layer-by-layer deposition, local differences in structure and composition, as indicated in previous work (Akiva-Tal et al., 2011; Bentov et al., 2010) could provide vital clues for the stabilization mechanism of ACC.

In order to better understand the thermodynamic stability and structure of gastrolith mineral in relationship to its biological formation history and purpose, in this study we performed spatially-resolved analysis on whole pre-molt gastrolith slices or cross-sections. To obtain a complete chemical, structural and morphological description of the gastrolith at different length scales, Raman spectroscopy and synchrotron small-and wide angle X-ray scattering (SAXS/WAXS) were combined with light microscopy imaging, high-resolution scanning electron microscopy (SEM) and EDS analysis. Results show that the structure as well as the content of inorganic phosphate, chitin, protein and citrate or PEP show layer-to-layer variations. Such a distribution indicates that the remarkable stability of the ACC is not governed by only one of these compounds, but that depending on the specific layer investigated, different stabilizing agents are involved.

2. Materials and methods

2.1. Preparation of the gastrolith cross-sections

Pre-molt gastroliths were extracted from the animals reared at BGU, and cleaned using distilled water. For SAXS/WAXS and light microscopy, samples were embedded in epoxy-resin (EPOFIX™) and sectioned using a diamond knife producing 0.7–1.0 mm thick slides, concomitantly cooled by ethylene glycol to prevent crystallization. Additionally, for chemical and structural characterization by Raman and SEM, gastrolith cross-sections were prepared by cutting the gastrolith with a scalpel blade while frozen by liquid nitrogen.

2.2. Light microscopy

Light microscopy images of gastrolith slides were taken by a Leica DM RXA2 microscope at a magnification of 2.5×. For visualization of the total gastrolith, images of different (overlapping) regions were combined afterwards using Microsoft Powerpoint™.

2.3. Scanning electron microscopy + energy dispersive X-ray spectroscopy

Scanning electron micrographs were obtained with a Jeol JSM7500F. Images were acquired at an acceleration voltage of 2 kV and a working distance (WD) of about 8 mm, using a through-the-lens secondary electron detector. The freshly exposed surfaces of the samples were coated with a layer of 2–3 nm of Pt prior to the investigations. Analytical information was obtained at 15 kV and at the same WD used for imaging through an Oxford Inca Energy Dispersive Spectroscopy System (EDS) using an X-Max™ silicon drift detector (spatial resolution ~ 2 μm). Using this equipment we were able to detect less than 2 mol% of P. Particle size measurements on SEM-images were performed using ImageJ™ software.

2.4. Raman spectroscopy

Raman spectra were collected with a confocal Raman microscope (α300; WITec) equipped with a Nikon objective (20×) a laser excitation wavelength of 532 nm and a spatial resolution of ~700 nm. Spectra were acquired with a CCD camera (DV401-BV; Andor) behind a spectrometer (UHTS 300; WITec) with a spectral resolution of 3 cm^{-1}. For the line scans, light microscopy images and corresponding spectra were taken every 50 μm. Data analysis (subtracting background, fitting area beneath specificated peaks) was done using WITec™ software. A rough estimation of P levels by Raman was done by comparison of the area of the ν_1 of PO_4^{3-} (~960 cm^{-1}) with the ν_1 of CO_3^{2-} (~1080 cm^{-1}). As a standard, synthetic P-containing ACC (ACCP) was prepared by adding a concentrated $CaCl_2$ solution to a 98:2 mixture of Na_2CO_3 and Na_2HPO_4. From inductively coupled plasma optical emission spectrometry (ICP–OES) analysis, 1.6 mol% of P was measured inside the P-ACC standard.

2.5. Synchrotron small- and wide angle X-ray scattering and X-ray diffraction measurements

Small and wide-angle X-ray scattering (SAXS + WAXS) measurements on embedded gastrolith slices were performed at the μ-Spot beamline (BESSY II storage ring, Helmholtz-Zentrum Berlin) (Paris et al., 2007) using a multilayer monochromator and spot size of 100 μm. Radially averaged scattering patterns were obtained using FIT2D™ software and corrected for sample thickness (transmission), intensity of X-ray beam and background. For quantitative peak area determination of the WAXS patterns, the two amorphous signals of ACC (AMO1 at $q \cong 22$ nm^{-1}, AMO2 at $q \cong 32$ nm^{-1}) and the α-chitin (1 1 0) at $q \cong 14$ nm^{-1} were fitted by gaussian curves using Origin™ Software.