Journal of Structural Biology 190 (2015) 291–303

Contents lists available at ScienceDirect

Journal of Structural Biology

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

Amorphous calcium carbonate controls avian eggshell mineralization: A new paradigm for understanding rapid eggshell calcification

Alejandro B. Rodríguez-Navarro [a,*], Pauline Marie [b], Yves Nys [b], Maxwell T. Hincke [c], Joel Gautron [b]

[a] Departmento de Mineralogia y Petrologia, Universidad de Granada, 18071 Granada, Spain
[b] INRA, UR83 Recherches Avicoles, F-37380 Nouzilly, France
[c] Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa K1H 8M5, Canada

ARTICLE INFO

Article history:
Received 12 March 2015
Received in revised form 21 April 2015
Accepted 22 April 2015
Available online 28 April 2015

Keywords:
Biomineralization
Laying hens
Eggshell
Amorphous calcium carbonate
Calcite

ABSTRACT

Avian eggshell mineralization is the fastest biogenic calcification process known in nature. How this is achieved while producing a highly crystalline material composed of large calcite columnar single crystals remains largely unknown. Here we report that eggshell mineral originates from the accumulation of flat disk-shaped amorphous calcium carbonate (ACC) particles on specific organic sites on the eggshell membrane, which are rich in proteins and sulfated proteoglycans. These structures known as mammillary cores promote the nucleation and stabilization of a amorphous calcium carbonate with calcitic short range order which predetermine the calcite composition of the mature eggshell. The amorphous nature of the precursor phase was confirmed by the diffuse scattering of X-rays and electrons. The nascent calcitic short-range order of this transient mineral phase was revealed by infrared spectroscopy and HRTEM. The ACC mineral deposited around the mammillary core sites progressively transforms directly into calcite crystals without the occurrence of any intermediate phase. Ionic speciation data suggest that the uterine fluid is equilibrated with amorphous calcium carbonate, throughout the duration of eggshell mineralization process, supporting that this mineral phase is constantly forming at the shell mineralization front. On the other hand, the transient amorphous calcium carbonate mineral deposits, as well as the calcite crystals into which they are converted, form by the ordered aggregation of nanoparticles that support the rapid mineralization of the eggshell. The results of this study alter our current understanding of avian eggshell calcification and provide new insights into the genesis and formation of calcium carbonate biominerals in vertebrates.

1. Introduction

Polymorphs of calcium carbonate are among the most common materials used by organisms for many different functions, especially for building highly sophisticated protective mineral structures (i.e., mollusk shell, avian eggshell (Addadi and Weiner, 1992; Nys et al., 1999)). The formation and properties of biomineralized structures have been extensively studied. As a result, our understanding of certain aspects of biomineralization and crystal growth mechanisms has radically changed in the last decade (Addadi et al., 2003; Banfield et al., 2000; Weiner and Addadi, 2011). In particular, the importance and ubiquity of amorphous mineral phases have been demonstrated in several organisms, in which they are used either as calcium storage deposits (i.e. crustaceans) or as a precursor transient mineral phase during the early stages of tissue calcification (i.e. sea urchin spicules) (Addadi et al., 2003). The amorphous mineral form is more soluble and reactive than its crystalline counterpart and can be used as a temporary calcium store, and solubilized as needed to supply calcium for rapid calcification (Ziegler, 1994; Levi-Kalisman et al., 2002; Luquet, 2012). The role of amorphous calcium carbonate (ACC) as a transient phase was first demonstrated during the initial stages of formation of the sea urchin spicule and the larval mollusk shell; in these invertebrate organisms, calcification is preceded by the formation of ACC as a transient phase that is later converted into more stable crystalline mineral phases (i.e., calcite, aragonite; Addadi et al., 2003; Beniash et al., 1997; Weiss et al., 2002). An important advantage is that amorphous phases can more easily adopt the complex morphologies found in biomineral structures, which are more difficult to attain by a single crystal; this recognition has inspired the development of new methods to fabricate highly sophisticated single crystal materials (Aizenberg et al., 2003; Fratzl et al., 2010). Additionally, amorphous phases can grow more

* Corresponding author.
E-mail address: anava@ugr.es (A.B. Rodríguez-Navarro).

http://dx.doi.org/10.1016/j.jsb.2015.04.014
1047-8477/

rapidly than crystalline ones (Raiteri and Gale, 2010; Smeets et al., 2015).

Avian eggshell formation is another biomineralization model system in which the presence of amorphous mineral phases could be highly relevant, although it has never been detected. The eggshell is sequentially assembled as the egg traverses the oviduct (Fernandez et al., 2001; Nys et al., 1999). The shell formation process can be divided into three stages (initiation, linear growth and termination). At the initiation stage, a quasi-periodic array of organic-rich nucleation sites are secreted on the outer fibers of the eggshell membranes, beginning at approximately 4.5 h post-ovulation (p.o.), when the egg briefly passes through the red isthmus, and then enters the uterus (the shell gland) where mineralization at these sites is initiated during several hours. During the subsequent linear growth stage (between 10 and 22 h p.o., while the forming egg is fully inflated and rotates in the uterus), the eggshell is rapidly mineralized until shell deposition is actively arrested at about 22 h p.o., two hours before oviposition (expulsion) (Nys et al., 1999). During the entire process, the egg is bathed in the uterine fluid, an acellular milieu which contains all necessary organic and inorganic precursors for eggshell formation. Shell mineralization requires the continuous supply of large amounts of calcium and carbonate ions from the uterine fluid, which are derived from the blood stream via trans-epithelial transport across the uterine gland cells (Jonchere et al., 2012). Ionic transporters, actively involved in the secretion of calcium, and carbonic anhydrase, which catalyzes the hydration of CO_2 to HCO_3^-, are highly overexpressed in the uterus gland cells during eggshell mineralization (Brionne et al., 2014; Jonchere et al., 2012). The organic matrix of the eggshell (proteins, polysaccharides and proteoglycans), is also concomitantly secreted into the uterine fluid (Brionne et al., 2014; Gautron et al., 1997). As in other biomineralization processes, the organic matrix is believed to play a key role in the modulation of shell formation, since at each stage a particular profile of specific proteins are present in the uterine fluid (Gautron et al., 1997). *In situ* and *in vitro* experiments reveal that these organic matrix components modulate the precipitation of calcium carbonate, including polymorph selection, and demonstrate that there is a functional correlation of the organic composition of the uterine fluid with each stage of the calcification process (Gautron et al., 1997; Hernandez-Hernandez et al., 2008). Moreover, during eggshell mineralization, the genes encoding for these proteins are selectively and highly expressed (Brionne et al., 2014).

Eggshell calcium carbonate precipitates from the uterine fluid, which is highly supersaturated with respect to calcite, the only mineral constituent of the mature eggshell (Nys et al., 1991). Surprisingly, even under these extreme conditions, eggshell mineralization occurs under precise control and produces a material with a well-defined ultrastructural organization (consisting of large columnar calcite crystal units) and monomineral composition (only calcite). Furthermore, the mineralization rate is modulated precisely during the phases of initiation, rapid growth and termination of eggshell formation (Nys et al., 2004). Though there many studies analyzing different aspects of the process of eggshell calcification (see above), there is no detailed study of the evolution of the mineralogy and crystallinity of the eggshell at the early stages of its calcification, and during the phase of linear deposition. These stages are of particular interest as other mineral phases different from calcite may be present. In particular, the formation of amorphous calcium carbonate (ACC) phases during eggshell formation has been recently implied. For instance, molecular dynamic simulations predict that one specific eggshell matrix protein (ovocleidin-17) could catalyze the transformation of ACC to calcite (Freeman et al., 2011). In additional, *in vitro* experiments reveal that different egg proteins (ovalbumin), eggshell organic extracts and mixtures of matrix proteins can induce the formation and stabilization of ACC as well as other metastable forms of calcium carbonate (vaterite) (Lakshminarayanan et al., 2006; Pipich et al., 2008; Wang et al., 2010; Wolf et al., 2011). Amorphous or non-crystalline solids might have short range order but lack translational periodicity or long range order characteristic of crystalline materials and therefore do not diffract X-rays (De Graef and McHenry, 2012). Consequently, amorphous mineral phases (i.e., ACC) are difficult to detect with traditional techniques used for mineral characterization (i.e., X-ray diffraction) and can be easily overlooked. Furthermore, the amorphous nature of mineral is often ambiguously defined as it depends on the structural resolution of analytical technique used for its characterization (optical microscopy, X-ray diffraction, infrared spectroscopy, X-ray absorption spectroscopy, TEM; Mahamid et al., 2008; Levi-Kalisman et al., 2002; Addadi et al., 2003; Gago-Duport et al., 2008).

In this work, the evolution of the mineralogy and crystallinity of eggshell mineral during its formation process have been reexamined using complementary analytical techniques (i.e., X-ray diffraction, infrared spectroscopy and HRTEM) which are able to detect and characterize in detail amorphous or poorly crystalline mineral phases (Addadi et al., 2003; Politi et al., 2006; Gueta et al., 2007; Poduska et al., 2010). These techniques provide information about the local structural conformation of amorphous or nanocrystalline materials at different level, enabling the detection of any incipient short range order in precursor amorphous phases which might predetermine the crystalline phases into which they will ultimately convert (Addadi et al., 2003; Cartwright et al., 2012) and help us understand the mechanisms controlling calcium carbonate precipitation in this complex biomaterial. In this report, we demonstrate for the first time the occurrence of a transient amorphous calcium carbonate mineral with calcitic short range order, during eggshell calcification, and reveal the importance of this mineral phase to explain the unique characteristics of eggshell mineralization.

2. Materials and methods

2.1. Ethical statement, animals handling and housing

All experiments, including all animal handling protocols, were carried out in accordance with the European Communities Council Directives concerning the practice for the care and Use of Animals for Scientific purposes and the French ministerial on Animal experimentation under the supervision of authorized scientists (authorization # 7323, delivered by the DDPP, direction départementale de la protection des populations, d'Indre et Loire). The experimental unit UE-PEAT 1295 where the birds were kept has authorization for rearing birds and for the euthanasia of experimental animals (decree N° B37-175-1 of August 28th 2012 delivered by the Préfecture d'Indre et Loire following the inspection of the Department Direction of Veterinary Services). The protocol was approved by an ethical committee (comité d'éthique de Val de Loire, officially registered under number 19 of the French national ethic committee for animal experimentation) under the authorization number 00159.02.

2.2. Materials

Brown egg laying hens (48–50 weeks old, ISA Hendrix, France) were caged individually and subjected to a cycle of 14 h of light/10 h of night. Each cage was equipped with a device for automatic recording of the time of oviposition (egg expulsion). Hens were fed a layer mash *ad libitum* as recommended by the Institut National de la Recherche Agronomique (INRA). Eggshell samples were