Avian eggshell formation: Presence of amorphous calcium carbonate associated with changes in some organic matrix proteins during initiation of mineralisation

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We have studied eggshell formation mechanisms in order to identify functional candidates involved in the process of mineralisation and which contribute to the reinforcement of eggshell quality. Eggshell is a biomineral made of 95% calcium carbonate (calcite) and 3.5% organic matrix (proteins and proteoglycans). This structure is the consequence of controlled interactions between both mineral and organic matrix constituents resulting in a highly ordered structure of the eggshell, with unique mechanical properties. Using *in situ* physical methods, we have recently observed that calcium carbonate was primary deposited on transient Amorphous Calcium Carbonate (ACC) as a more soluble and reactive form (Rodriguez-Navarro *et al.*, 2015), leading to the hypothesis that some eggshell matrix proteins might play a pivotal role to stabilize the ACC, to influence the crystal polymorph and therefore the shell ultrastructure. We have used a quantitative proteomic approach at 4 pivotal stages of shell mineralisation and we have identified 175 proteins with variation of abundance during the calcification process (Marie *et al.*, 2015). Functional analysis has allowed the characterization of 77 putative functional proteins of the shell calcification. We confirmed the important role of lysozyme, ovotransferrin, ovocleidin-17 and ovocleidin-116 for the shell calcification process, and described and characterized novel major calcium binding proteins and proteoglycans core proteins. We suggested that OVAL and OC-17 play a role in the stabilization of ACC. Finally, we reported proteins involved in the regulation of proteins driving the mineralisation.

Keywords: eggshell; formation; amorphous calcium carbonate; matrix proteins; mineralisation.

Introduction

The calcified chicken eggshell is a natural envelope which protects the developing embryo against physical damage and microbial assaults, and ensures that the nutritious table egg remains free of pathogens. It is a well-defined structural polycrystalline organisation composed of 95% calcium carbonate (calcite polymorph), 1.5% water and 3.5% proteins, polysaccharides and proteoglycans constituting its organic matrix (Nys *et al.*, 2004). Avian eggshell is a porous mineral layer resulting from the sequential deposition of its different zones during precisely defined phases in the distal oviduct. Five hours after the ovulation of the yolk, eggshell calcification is initiated in the red isthmus/uterus part, and lasts about 19 hours (Nys *et al.* 2004). The mineralisation process starts by

heterogeneous nucleation on specific sites (organic cores also called mammillary knobs), located on the surface of the outer eggshell membranes. The initiation of shell formation is followed by linear deposition of mineral until the process is inhibited. These distinct phases of calcification result in the formation of different layers: the inner mammillary cones, the palisade layer and the cuticle. Amorphous calcium carbonate (ACC) is now recognized as an early and transient non-crystalline precursor phase of calcite or aragonite in the $CaCO_3$ calcified structures produced by many invertebrates (Addadi and Weiner, 2014). It represents an active and highly reactive transient mineral phase to allow the growth of single crystals with very complex shapes. In the case of the eggshell, the presence of ACC was reported recently and has constituted a major advance to explain how the eggshell biomineralisation events are temporally / spatially nucleated and regulated (Rodriguez-Navarro *et al.,* 2015). Calcite crystals are formed by the aggregation of ACC particles that support the rapid mineralisation of the eggshell and there is evidence that this non-crystalline form of calcium carbonate is present throughout the phases of shell formation. The ACC mineral firstly accumulates on whole surface of eggshell membranes then is reallocated on specific nucleation sites (mammillary knobs). ACC dissolves rapidly, providing a continuous supply of ions to initially form calcite crystals on specific nucleation sites. These units coalesce to form larger crystals in the mammillary layer, and then during the following rapid growth phase they form the compact shell palisade layer made of columnar crystals with preferred orientation (Rodriguez-Navarro *et al.*,2015).

During these phases, matrix proteins probably play a key role to stabilize the ACC, influence the crystal polymorph and the crystal morphology which leads to the eggshell ultrastructure and its resulting mechanical properties. In a recent study, we have used quantitative proteomics and additional bioinformatic analysis to provide a comprehensive report on the potential role of eggshell matrix proteins involved in the primary nucleation (ACC deposition), initiation of mineralisation (calcite crystal formation by aggregation of ACC), formation of larger calcite crystals and then development of the columnar palisade layer with preferred calcite orientation (Marie *et al.*, 2015).

Materials and methods

The methods are described in Marie et al, 2015. Sixty brown-egg laying hens (ISA-Hendrix,) were caged individually and oviposition time was recorded. Forming eggs were collected at the initial phase of eggshell mineralisation (5, 6 and 7 hours after ovulation, p.o.) when the nucleation sites appear and early mineralisation starts, or during the linear growth phase of rapid calcification (16 hours p.o.).. Eggshell matrix proteins were extracted as described in Gautron *et al.*, 2001, with slight modifications (Marie *et al.*,2015). Six samples collected at the same time point were pooled in equal amounts for each time (5h p.o., 6h p.o., 7h p.o. and 16h p.o.). The four pooled samples were fractionated on SDS-PAGE gel and proteins were in-gel digested with bovine trypsin before to be analysed by nanoscale liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) (Labas *et al.*, 2015). Two different methods were applied to discern the relative abundance of the proteins: 1) emPAI from the proteins was calculated at each stage of sample collection (5, 6, 7 and 16 hours p.o.) to classify the relative abundance of the different proteins within individual stages and to determine the most abundant proteins. 2) In a second approach, GeLC-MS/MS analyses, combined with label free quantitative analysis based on a spectral counting method, were used to determine quantitative values depending on the stage of mineralisation. Hierarchical clustering was used to determine the different groups of proteins with different abundances according to time points of mineralisation. Conserved and functional domains were extracted from protein sequences to determine the potential functions of the matrix proteins.

Results and discussion

Forming eggshell samples were collected at four stages during the eggshell formation which are pivotal to establish the eggshell ultrastructure and crystallographic texture. They corresponded to the initial stage dominated by amorphous calcium carbonate (ACC) deposition on eggshell membranes at 5 hours post ovulation (5h p.o.), its progressive transformation to form calcite aggregates on

mammillary knobs surrounded by ACC particle (6h p.o.) and the growth of large calcite units surrounded by ACC (7h p.o.). The last stage corresponded to the formation of the columnar calcite crystals with the progressive development of preferred crystal orientation (16h p.o.) (Rodriguez-Navarro *et al.,* 2015). We have extracted proteins from these forming eggshell samples and obtained quantitative data for 216 proteins and determined 175 proteins with differential abundance according to the four time points of the shell calcification process. We paid particular attention to 77 of them, which exhibited potential functions associated with shell mineralisation (Marie *et al.,* 2015). We distinguished proteins having a direct involvement in shell mineralisation (mineralising proteins, able to bind calcium or divalent ions) from proteins indirectly involved into the calcification process through regulation of the proteins driving mineralisation.
We classified these proteins into various groups corresponding to the different events of shell mineralisation.

Overabundant proteins during primary events of shell calcification

Proteins described in this group corresponded to 27 proteins which were overabundant at the earliest stages of shell formation when a massive accumulation of ACC particles occurs on the entire surface of the forming shell. Eight of them might be associated to the mineralisation process (Marie *et al.,* 2015). We paid particular attention to lysozyme (LYZ) and ovotransferrin (OVOT), previously identified in eggshells and shown to modify crystal morphology *in vitro* (Hincke *et al.,* 2000; Gautron *et al.,* 2001). LYZ is the most abundant protein, which was observed in our study at the stages of shell calcification. Calcite crystals grown *in vitro* in the presence of lysozyme exhibited altered crystal morphology and its role in the stabilization of ACC has been suggested but remains controversial.
A similar role might be ascribed to OVOT which affects calcite crystal morphology at low concentrations (Gautron *et al.,* 2001) and is the second abundant protein at this stage, and to hemopexin (HPX) which exhibits divalent ions binding properties.

Proteoglycans are major actors in calcification in biomineralisation processes and various representatives have been detected in eggshells (Fernandez *et al.,* 1997). These macromolecules combine a protein core with negatively charged complex polysaccharides, which highly interact with calcium. They are thought to participate in eggshell mineralisation. We identified Hyaluronan and Proteoglycan LiNk protein 3 (HAPLN3) in this group as a protein with proteoglycan binding properties. HAPLN3 is 9th and 11th most abundant protein at 5 and 6 hours p.o., indicating a potential role in the mineralisation process.

In the second group involved in protein regulation, ovomucoid (OVM) is a protease inhibitor protein containing Kazal-like domains (Réhault-Godbert *et al.,* 2011), which were overabundant at 5 hours p.o. and remains an abundant eggshell matrix protein at all stages of shell mineralisation. OVM would have an indirect role in the calcification process by controlling the activity of eggshell matrix proteins, either by degrading proteins or by modifying the maturation of precursor proteins.

Overabundant proteins during ACC transformation into calcite aggregates

This stage corresponds to 6h p.o., when the forming eggshell weight has increased by 0.1 g to reach 0.28 g. This increase is the result of ACC accumulation and its progressive transformation to form calcite aggregates on the specific organic mammillary knobs which are the “nucleation sites” of the calcitic eggshell (Rodriguez-Navarro *et al.,* 2015). Thirteen proteins were found to be overabundant at this stage, but the functions of only three of them were related to shell mineralisation (Marie *et al.,* 2015). Ovocleidin-17 is notable as this overabundant protein at this stage is also the 3rd most abundant matrix protein whatever the stage. Molecular dynamic simulations suggest that OC-17 can catalyse the transformation of ACC into calcite (Freeman *et al.,* 2010). In addition, other C-type lectin proteins were identified in organic matrices from other biomineralisation systems. Of these, the role of spicule matrix protein 50 (SM-50) on calcium carbonate mineralisation has been investigated (Rao *et al.,* 2013) and the glycine rich domain of the protein was reported to be involved in ACC stabilization. This evidence and the fact that this protein is overabundant at this stage reinforced its involvement in the transformation of ACC mineral deposits into calcite aggregates.

Overabundant proteins when larger calcite crystal units are formed

At this step, larger calcite crystal units are growing on mammillary knobs to form the mammillary layer of the eggshell. ACC is suspected to remain present at the mineralisation front during this stage (Rodriguez-Navarro *et al.,* 2015). A total of 47 proteins were found to be overabundant at this particular stage, 6 to 7 hours after ovulation and the functions of 16 of them showed properties suggesting possible involvement in shell mineralisation (Marie *et al.,* 2015). Amongst them is ovalbumin (OVAL), one of the most abundant eggshell matrix proteins at all stages of shell calcification (Marie *et al.,* 2015). A schematic representation of calcium carbonate mineralisation in the presence of ovalbumin has been proposed (Pipich *et al.,* 2008). Calcium ions are bound to the protein by complexation through acidic groups leading to protein rearrangements. The calcium cations are the starting points for the subsequent formation of ACC nuclei which then undergo a series of phase transitions to the stable crystalline polymorphs (Schwahn *et al.,* 2004). These observations, its high abundance in the shell, its overabundance when larger calcite crystals are formed are strong evidences that OVAL is one of the major proteins involved in shell formation and contributes to the formation of metastable ACC and to control calcite crystal morphology and size. A similar role could be ascribed to Milk Fat Globule-EGF factor 8 protein (MFGE8) which contains EGF-like calcium binding domain. Its presence as a fairly abundant protein at the different stages of shell calcification and the recent report that it is highly expressed in the uterus when a shell is undergoing calcification (Jonchère *et al.,* 2010; Brionne *et al.,* 2014) provides strong evidence in support of the functional role of this protein in the chicken eggshell calcification process. Ovocalyxin-32 (OCX32) is also present in this group (Marie *et al.,* 2015) and correlations between OCX32 gene polymorphisms and different eggshell quality phenotypes were reported (Dunn *et al.,* 2008, 2012; Fulton *et al.,* 2012; Takahashi *et al.,* 2010).

In this group, we also identified proteins potentially involved in the regulation of the activity of proteins driving mineralisation. The activities of these proteins might rely on an interaction with proteins directly involved in controlling the calcification process. Of particular interest are proteins involved in protein-protein interactions to ensure the proper folding of eggshell matrix proteins and regulation of their activity. Amongst them, clusterin (CLU), a chaperone protein, was identified in eggshell and was suspected to prevent the premature aggregation and precipitation of eggshell components during calcification (Mann *et al.,* 2003). We also identified LOC428451 as being overabundant in this group. This protein is able to remove phosphate groups, suggesting that it has an important role in the degree of phosphorylation of matrix proteins to modulate the kinetics of mineralisation (Hincke and St Maurice 2000).

Overabundant proteins during development of the columnar palisade layer with preferred orientation of calcite

We collected forming eggshell samples in the middle of this active phase (16h p.o.) and we revealed 70 overabundant proteins at this stage. The functions of 38 of them show properties suggesting a possible involvement in mineralisation (Marie *et al.,* 2015).

Albumin (ALB) binds calcium and is one of the major eggshell matrix proteins whatever the stage of shell formation (Marie *et al.,* 2015). Core proteins of shell proteoglycans suspected to play a role in shell biomineralisation at this stage are also reported. Numerous pieces of evidence suggest that one of the most important proteins is ovocleidin-116 (OC-116) which corresponds to the protein core of a dermatan sulphate proteoglycan mainly present in the palisade region of the shell (Hincke *et al.,* 1999). Another core protein of proteoglycan is glypican-4 (GPC4), which belongs to the glypican family which are heparan sulphate proteoglycans.

We identified additional proteins involved in the regulation of the activity of proteins driving mineralisation as previously described. We reported the overabundance of 12 proteases and protease inhibitors that could potentially control the calcification process, either by degrading proteins or by modifying the processing of protein maturation (Marie *et al.,* 2015). Cystatin C (CST3) appears to be an important candidate for protein regulation as it is abundant throughout all stages of mineralisation. We have also revealed several molecular chaperones involved in protein-protein interactions to ensure the proper folding of eggshell matrix proteins and the regulation of their activity (Marie *et al.,* 2015).

Ovocalyxin-21 (OCX21), an eggshell matrix protein that contains a BRICHOS domain associated to chaperone molecules (Gautron and Nys, 2007), appears to be the most promising protein candidate of the group of molecular chaperones. This protein is particularly abundant at this stage in accordance with its identification as having the highest emPAI in a proteomic analysis of the soluble organic matrix of the entire chicken eggshell (Mann *et al.,* 2006). Peptidylprolyl isomerase B (PPIB) belongs to the same protein family and is also highly abundant at this stage.

Overabundant proteins throughout the stages of shell calcification

This group differed from the previous ones by the fact that the protein level increased at an early stage and then plateaued throughout the process of shell deposition. These 18 proteins become overabundant as soon as calcite is formed (6-7h p.o.) and remain overabundant at the later stages when large calcite units are deposited (7-16h p.o.). The function of 12 of them could be associated with the mineralisation process (Marie *et al.,*2015).

Amongst them are 2 calcium binding proteins. EGF-like repeats and Discoidin I-Like domains 3 (EDIL3) contains one EFG-like calcium binding domain and is of major importance at all stages of mineralisation. Its presence as a major eggshell matrix protein was thoroughly reported by proteomics (Mann *et al.,* 2006; Miksik *et al.,* 2010; Sun *et al.,* 2013), and its transcript was strongly expressed in the chicken uterus (Brionne *et al.,* 2014). Consequently, EDIL3 is believed to play a major role in the eggshell calcification. Nucleobindin 2 (NUCB2) might be another candidate which is pertinent to eggshell calcite mineralisation. This protein, containing two EF-hand domains, was reported to be over-expressed when a shell is calcifying (Jonchère *et al.,* 2010).

Also present in this group is Ovocalyxin-25 (OCX25), previously identified as an eggshell matrix protein containing 2 protease inhibitors domains (Gautron and Nys, 2007; Rose-Martel *et al.,* 2012), and consequently suspected to be involved in the regulation of the activity of proteins driving mineralisation.

Conclusions

A total of 175 proteins with various abundances during the events of mineralisation which are pivotal for the control of eggshell crystallographic ultrastructure were identified (Marie *et al.,* 2015). The potential functions relative to mineralisation of 77 proteins extracted from the forming eggshell at three initial stages of shell calcification (1) deposition of a metastable amorphous calcium carbonate over the entire surface of the eggshell membranes, (2) redistribution of ACC on organic nucleation sites to form aggregates of calcite microcrystals, (3) enlargement of calcite crystal units to form the mammillary layer and the final stage (4) development of the columnar calcite units with preferred orientation in the compact eggshell layer, were explored. We have selected 20 matrix proteins with high emPAI values and changes during the different shell formation stages and suspected to have predominant roles in the control of the different stages of shell calcification. Characterisation of their function in the process of shell mineralisation will require further studies to define their interactions with the mineral phase.

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