Relative influences of solution composition and presence of intracrystalline proteins on magnesium incorporation in calcium carbonate minerals: Insight into vital effects

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[1] Biogenic calcites may contain considerable magnesium concentrations, significantly higher than those observed in inorganic calcites. Control of ion concentrations in the calcifying space by transport systems and properties of the organic matrix of mineralization are probably involved in the incorporation of high magnesium quantities in biogenic calcites, but their relative effects have never been quantified. In vitro precipitation experiments performed at different Mg/Ca ratios in the solution and in the presence of soluble organic matrix macromolecules (SOM) extracted from sea urchin tests and spines showed that, at a constant temperature, magnesium incorporation in the precipitated minerals was mainly dependent on the Mg/Ca ratio of the solution. However, a significant increase in magnesium incorporation was observed in the presence of SOM compared with control experiments. Furthermore, this effect was more pronounced with SOM extracted from the test, which was richer in magnesium than the spines. According to SEM observations, amorphous calcium carbonate was precipitated at high $Mg/Ca_{solution}$. The observed predominant effect of $Mg/Ca_{solution}$, probably mediated in vivo by ion transport to and from the calcifying space, was suggested to induce and stabilize a transient magnesium-rich amorphous phase essential to the formation of high magnesium calcites. Aspartic acid rich proteins, shown to be more abundant in the test than in the spine matrix, further stabilize this amorphous phase. The involvement of the organic matrix in this process can explain the observation that sympatric organisms or even different skeletal elements of the same individual present different skeletal magnesium concentrations.

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1. Introduction

[2] Marine invertebrates produce calcite skeletons with a large variety of chemical compositions and morphologies. These biogenic calcites are composed of a dominant mineral phase including an intimately associated organic phase. The mineral phase may contain considerable concentrations of magnesium, ranging from a few percent up to 43.5 mol % $MgCO_3$, as the major ion substituted to calcium in the mineral lattice [*Chave*, 1954; *Schroeder et al.*, 1969]. Biogenic calcites may display higher magnesium concentrations than inorganically deposited calcites, produced either in nature or in the laboratory, which typically include less than 10 mol % $MgCO_3$ with extreme values up to 21 mol % [*Raz et al.*, 2000; *Cheng et al.*, 2007; *Wang et al.*, 2009].

[3] The organic phase, the so-called organic matrix of mineralization, is mainly composed of proteins and glycans. Despite its usually low concentration, the organic matrix of mineralization plays an essential role in nucleation and crystal growth control. It affects the morphology of the deposited mineral and may determine the precipitated polymorph [*Lowenstam and Weiner*, 1989; *Simkiss and Wilbur*, 1989; *Addadi and Weiner*, 1992; *Falini et al.*, 1996]. In particular, proteins enriched in the carboxyl-rich acidic amino acids aspartate and glutamate are thought to play a significant role in the modulation of biomineral formation [*Addadi and Weiner*, 1985, 1992; *Politi et al.*, 2007; *Stephenson et al.*, 2008].

[4] Magnesium concentrations in marine biogenic calcites are known to vary according to environmental parameters, such as temperature, salinity, and Mg/Ca ratio in seawater, which prompted the use of calcite skeletons as paleorecorders [*Clarke and Wheeler*, 1922; *Lea et al.*, 1999; *Lea*, 2003; *Ries*, 2004; *Borremans et al.*, 2009; *Hermans et al.*,

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2010]. However, magnesium concentrations in the skeleton of closely related taxa living in the same environment can be very different, indicating that biological factors, the so-called "vital effects," are also involved [*Weiner and Dove*, 2003; *Bentov and Erez*, 2006]. The nature of these effects is, in most cases, poorly understood. Organic molecules and a biological control of ion concentrations in the calcifying space were suggested to be involved [*Bentov and Erez*, 2006], but the relative effects of these parameters are still unexplored.

[5] At a constant temperature, the Mg/Ca ratio of in vitro deposited calcite is affected by the same ratio in the solution. Skeletal Mg/Ca ratios of calcifying organisms grown under experimental conditions were also shown to be directly, but not linearly, controlled by the Mg/Ca ratio of seawater [*Lorens and Bender*, 1980; *Ries*, 2004]. Biogenic calcites form through a transient phase of amorphous calcium carbonate [*Aizenberg et al.*, 1996; *Beniash et al.*, 1997, 1999; *Politi et al.*, 2004] which has been suggested to affect the magnesium signature of the final crystal [*Loste et al.*, 2003; *Wang et al.*, 2009]. *Loste et al.* [2003] showed that the Mg/Ca ratio of transient amorphous calcium carbonate precipitated in vitro was related to the Mg/Ca ratio of the precipitation solution and determined the magnesium concentration in the resulting crystals. *Han et al.* [2005] and *Cheng et al.* [2007] showed a nearly linear increase of magnesium content of in vitro precipitated calcium carbonate minerals with the Mg/Ca ratio of the precipitation solution.

[6] The ability of synthetic organic molecules to affect the magnesium concentration in calcites has been demonstrated by in vitro precipitation experiments. *Kitano and Kanamori* [1966] showed that sodium citrate and malate enhance magnesium incorporation into calcite and reduce the formation of aragonite, which is the dominant precipitated mineral in inorganic magnesium-rich solutions. Biomimetic peptides and polypeptides (aspartic acids) induce similar promoting effects on magnesium incorporation into calcite [*Cheng et al.*, 2007; *Stephenson et al.*, 2008]. However, *Falini et al.* [1994] reported that magnesium incorporation into crystals grown in vitro in the presence of poly-L-aspartate was not enhanced, although this compound promoted the formation of calcite in magnesium-rich solution.

[7] Very few studies investigated the effect of genuine organic matrix molecules on magnesium incorporation. According to these, the presence of organic matrix had no or low impact on magnesium concentration in the precipitated mineral [*Raz et al.*, 2003; *Gayathri et al.*, 2007]. However, these studies used only a single matrix concentration and one of them used a single solution Mg/Ca ratio, preventing a firm conclusion.

[8] Sea urchins produce an intradermic skeleton of high-magnesium calcite. Magnesium concentrations in skeletons differ according to the taxa [*Weber*, 1969]. In a considered species, different skeletal elements may have contrasted magnesium concentrations [*Weber*, 1969]. For instance, the sea urchin test has a higher magnesium concentration than the spines in the same individual. This characteristic offers an interesting model to compare the effect of possibly differentiated organic matrices without specific bias. In the present study, we investigated the relative influences of the Mg/Ca ratio in the precipitation solution and the concentration and nature of genuine organic matrices on the morphology and Mg/Ca ratio of in vitro precipitated minerals. We showed that spine and test organic matrices induce different Mg/Ca ratios in the precipitated mineral.

2. Materials and Methods

2.1. Specimen Collection and Skeletal Cleaning

[9] Thirty adult *Paracentrotus lividus* specimens were collected by scuba diving in La Vesse (Bouches-du-Rhone, France). Their ambital diameters, measured using calipers, ranged from 44 to 61 mm with a mean (±standard deviation) of 50.6 ± 4.7 mm. They were dissected and stored at −20°C. The skeleton of spines and test were cleaned from associated tissues with 2.5% NaOCl (pro analysi) at 4°C on a rocking table, during respectively 3 and 24 h. Skeletal elements were rinsed three times in Milli Q water (Millipore). The cleaning procedure was checked by observation with a scanning electron microscope. Interambulacral plates of the disarticulated test and primary spines were selected and separately ground in an agate mortar. These homogeneous skeletal powders were stored at −20°C.

2.2. Macromolecule Extraction

[10] The intraskeletal organic matrix macromolecules were extracted from the mineral phase by decalcification using an ion exchange resin. The skeletal powder (8 g) suspended in Milli Q water was enclosed in a dialysis tube (Spectra/Por MWCO 3.5kD) placed in a Plexiglas cylinder. Dowex (50WX8, 50–100 mesh) ion exchange resin, prewashed with Milli Q water, was placed in the cylinder and Milli Q water containing 0.02% NaN_3 was added to fill it [*Albeck et al.*, 1996b]. The cylinder was continuously rotated in a propeller-like mode at 4°C. The resin and Milli Q water were changed daily. After complete decalcification of the skeletal powder, the content of the dialysis bag was dialyzed against Milli Q water for 3 days. The volume of the extract was then reduced to 2 mL by lyophilization. The soluble and insoluble materials were separated by three centrifugations at 18.000*g*, 4°C, for 15 min. This organic matrix extraction process was carried out in parallel in four cylinders, and the resulting extracts of soluble organic matrix (SOM) were combined, divided into aliquots frozen in liquid nitrogen, and stored at −80°C for further use.

2.3. Amino Acid Analysis

[11] Amino acid compositions of one aliquot of spine and test extracts were determined by Alphalyse A/S (Odense, Denmark). Samples were hydrolyzed under reduced pressure, in 6 N HCl, 0.1% phenol, and 0.1% thioglycolic acid at 110°C for 20 h. Identification and quantification of the amino acids took place on a BioChrom 30 amino acid analyzer using ion exchange chromatography, postcolumn derivatization with ninhydrin, and detection at two wavelengths, 570 and 440 nm. The common 20 amino acids, except tryptophan and cysteine, were determined. A known amount of the nonnatural amino acid norleucine (Nle) was added as an internal control standard. Recovery was always higher than 90%. Analysis for all amino acids were above the detection limit as determined on chromatograms except for lysine in test SOM.

Table 1. Amino Acid Composition (mol %) of Test and Spine Soluble Organic Matrices Used for in Vitro Crystallization Experiments

	Test	Spine
Asx	16.5	12.9
Thr	4.1	6.5
Ser	5.4	5.1
Glx	10.9	12
Gly	31.9	18.1
Ala	13.6	11
Val	4.4	4.9
Met	0.4	2.2
Ile	1.9	3.4
Leu	1.7	4.6
Tyr	1.7	2
Phe	5.5	4.3
His	0.4	1.1
Lys	0	1.1
Arg	0.9	1.9
Pro	0.7	9

2.4. Crystal Growth Experiments

[12] The crystallization experiments were performed in Nunc multidishes (24 wells, 15 mm diameter), with glass coverslips (13 mm diameter, VWR International) placed on the bottom of each well according to *Albeck et al.* [1993]. A total volume of 0.5 mL was introduced into each well: calcium chloride and magnesium chloride solutions (0.05 mol/L, pro analysi, Merck) and SOM extract were poured in amounts fitting with the desired protein concentrations (1, 5, and 10 μg/mL) and molar Mg/Ca ratios in the solution ($Mg/Ca_{solution}$ = 1:1; 3:1; 4:1; 5:1). Each well was sealed separately with aluminum foil and punctured with a needle (21G). The multidish was placed into a closed desiccator with a vial containing NH_4CO_3 (Merck, pro analysi) and covered with parafilm punctured with a needle. The crystallization was performed for 48 h at a constant temperature of 18°C. Control experiments were carried out in the absence of organic additive, in the absence of magnesium (at 0 and 10 μg/mL proteins), and with bovine serum albumin (BSA, 10 μg/mL) as a nonspecific protein. The experiment was also repeated without ammonium carbonate at $Mg/Ca_{solution}$ of 1:1 and 5:1, in the absence and presence of proteins (1 and 10 μg/mL) to check for magnesium and calcium adsorption on the coverslip. Elemental adsorption on the glass coverslip never exceeded 3% of experimental values for both elements, indicating that almost all of the measured calcium and magnesium were effectively in the mineral deposits. After 48 h, the glass coverslips were briefly rinsed with Milli Q water and air dried.

2.5. Scanning Electron Microscopy (SEM) Observations

[13] For each treatment, one slide was mounted on stub with double-sided carbon tape. To increase conductivity, the samples were surrounded with conductive silver glue and gold coated. The slides were observed on a JEOL JSM-6100 SEM.

2.6. High Resolution Inductively Coupled Plasma Mass Spectroscopy (HR-ICP-MS) Analysis

[14] For each treatment, minerals deposited on four slides were dissolved, separately, in 140 μL of HNO_3 (65%, suprapur, Merck) and 360 μL of Milli Q water in a glass crystallizer. This solution was diluted 20 times before analysis in a solution containing 1 μg/L In, which was used as internal standard. ^{26}Mg and ^{43}Ca were analyzed on a Thermo Finnigan Element 2. The analytical reproducibility ($2s$) was 0.43 and 1.54, and accuracy was 1.23% and 3.5% for ^{26}Mg and ^{43}Ca, respectively. A multielement calibration standard was prepared from certified single element stock solution. The certified reference material SLRS-4 was run for a quality check. Mg and Ca concentrations in the samples were converted to mol % $MgCO_3$.

2.7. Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) Analysis

[15] Six aliquots of 0.25 g of both spine and test skeletal powders were mineralized in a Milestone 1200mega microwave oven in 2.5 mL of HNO_3 and 1 mL of H_2O_2. The resulting solutions were filtered on a GF/A Whatman filter, brought to a final volume of 25 mL with Milli Q water (Millipore) and diluted 10 times in acidified water prior to analysis. Mg and Ca concentrations of the solutions were analyzed with an Iris Advantage (Thermo Jarrel Ash) ICP-AES apparatus. The calibration was achieved using artificial multielemental solutions made from certified monoelemental solutions (Merck), and certified reference material JCp-1 (coral) (Standard Geological Survey of Japan) was used for a quality check. Results for the certified reference material analysis were always within ±10% of the certified values.

2.8. Data Analyses

[16] All statistical analyses were carried out using the Systat 9 software. The significance level was fixed at 0.05. For each type of SOM (test and spine), the influence of $Mg/Ca_{solution}$ and protein concentration was assessed using forward stepwise multiple regression. The effects of 0 and 10 μg/mL concentrations and protein nature (BSA and both extracts) on the Mg/Ca of formed minerals were also compared using a two-way analysis of variance (ANOVA) (protein nature or absence and $Mg/Ca_{solution}$ as independent variables) and a Tukey post hoc test.

3. Results

3.1. Magnesium and Amino Acid Analysis of Spine and Test

[17] Mean magnesium concentrations (±standard deviation (SD), $n = 6$) in the spine and test were, respectively, 3.43 ± 0.04 and 9.84 ± 0.05 mol % $MgCO_3$. The amino acid compositions of the soluble organic matrix (SOM) of spines and tests are presented in Table 1. Test SOM showed a higher proportion of Asx and Gly, while spine SOM was richer in Pro.

3.2. Magnesium Concentrations of in Vitro Precipitated Minerals

[18] The magnesium concentrations of in vitro precipitated minerals varied significantly according to the $Mg/Ca_{solution}$ ratio ($p_{regression} < 10^{-4}$, Figure 1 and Table 2) and protein concentration (1, 5, and 10 μg/mL, $p_{regression} < 10^{-2}$, Figure 2 and Table 2). It is noteworthy that the mineral Mg/Ca ratio was maximal when concentrations of 5 μg/mL proteins were introduced. Whatever the sequence of introduction of the variables, the stepwise multiple

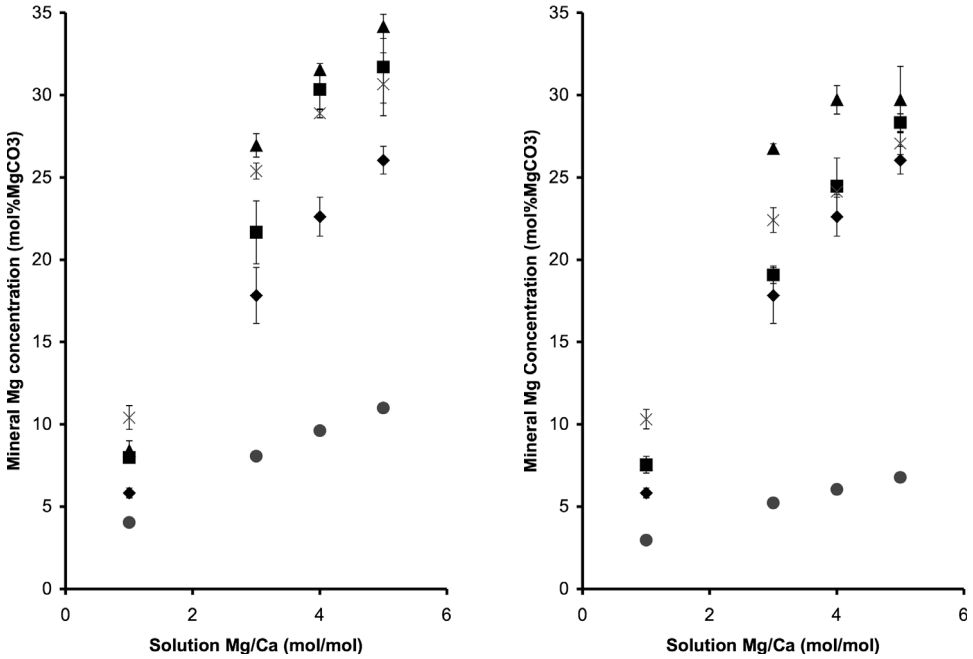

Figure 1. Mean magnesium concentration (±standard deviation, $n = 4$) in minerals precipitated in vitro in the presence of (a) test and (b) spine protein extracts, according to precipitation solution Mg/Ca and protein concentration (solid diamond, 0 μg/mL; solid square, 1 μg/mL; solid triangle, 5 μg/mL; cross, 10 μg/mL). Hypothetical skeletal $MgCO_3$ of sea urchins grown in different seawater Mg/Ca ratios (solid circle) were calculated from Ries' [*Ries*, 2004] algorithms for echinoid plate ($Mg/Ca_{skeleton} = (0.000719T + 0.0292)Mg/Ca_{SW}^{0.668}$) and spine ($Mg/Ca_{skeleton} = (0.000837T + 0.0155)Mg/Ca_{SW}^{0.538}$), at 18°C.

regressions indicated the predominant effect of the $Mg/Ca_{solution}$ ratio (explaining respectively 85% and 86% of the variation) compared to the effect of protein concentration (Table 2).

[19] The magnesium concentration of mineral deposits differed according to the nature of protein additives ($p_{2\text{-wayANOVA}} < 10^{-8}$, Figure 3). Test SOM induced higher magnesium concentrations in mineral deposits than spine SOM ($p_{Tukey} < 10^{-5}$). Deposits precipitated in the presence of either SOM extract showed higher magnesium concentrations than those precipitated in the presence of BSA or the absence of protein additives ($p_{Tukey} < 10^{-5}$). By contrast, magnesium concentrations in deposits precipitated in the presence of BSA or in the absence of additives did not differ ($p_{Tukey} = 0.79$).

3.3. Morphology of in Vitro Precipitated Minerals

[20] In the absence of magnesium and organic additive, rhombohedra presenting the classical {104} faces precipitated (Figure 4a). In the absence of magnesium but in the presence of 10 μg/mL SOM, crystals exhibited well-developed {104} faces terminated with rounded edges with steps (Figure 4b).

[21] In the presence of magnesium, the mineral shape was considerably modified. At low magnesium concentration (Mg/Ca = 1:1), crystals (ca. 100 μm) were elongated along the *c* axis and showed rough curved surfaces, parallel to the *c* axis, capped by rhombohedral faces (Figure 4c). Progressive changes in morphology were observed with increasing $Mg/Ca_{solution}$. Deposits 35–40 μm in length, formed in a 3:1 $Mg/Ca_{solution}$, presented peanut-like or dumbbell-shaped morphologies (Figures 4d and 4e). Polycrystalline aggregates of 50 μm diameter were also observed. At higher Mg/Ca ratios (4:1 and 5:1), a film covering the glass coverslip with small spherical particles of ~1 μm diameter was deposited (Figure 4f).

[22] Test and spine SOM also deeply affected the deposit morphology. These were rather similar with both extracts.

Table 2. Statistical Results of the Stepwise Multiple Regressions Between the Mg/Ca Ratio of in Vitro Precipitated Minerals and Possible Independent Variables

	Additional Organic Matrix	First Variable	Second Variable	Second Variable	R^2	Significance of Additional Variable
Mineral Mg/Ca	Spine	$Mg/Ca_{solution}$	–	–	0.85	$<10^{-4}$
		$Mg/Ca_{solution}$	Protein concentration	–	0.89	$<10^{-3}$
		$Mg/Ca_{solution}$	Protein concentration	Protein concentration^2	0.92	$<10^{-4}$
Mineral Mg/Ca	Test	$Mg/Ca_{solution}$	–	–	0.86	$<10^{-4}$
		$Mg/Ca_{solution}$	Protein concentration	–	0.88	$<10^{-2}$
		$Mg/Ca_{solution}$	Protein concentration	Protein concentration^2	0.92	$<10^{-4}$

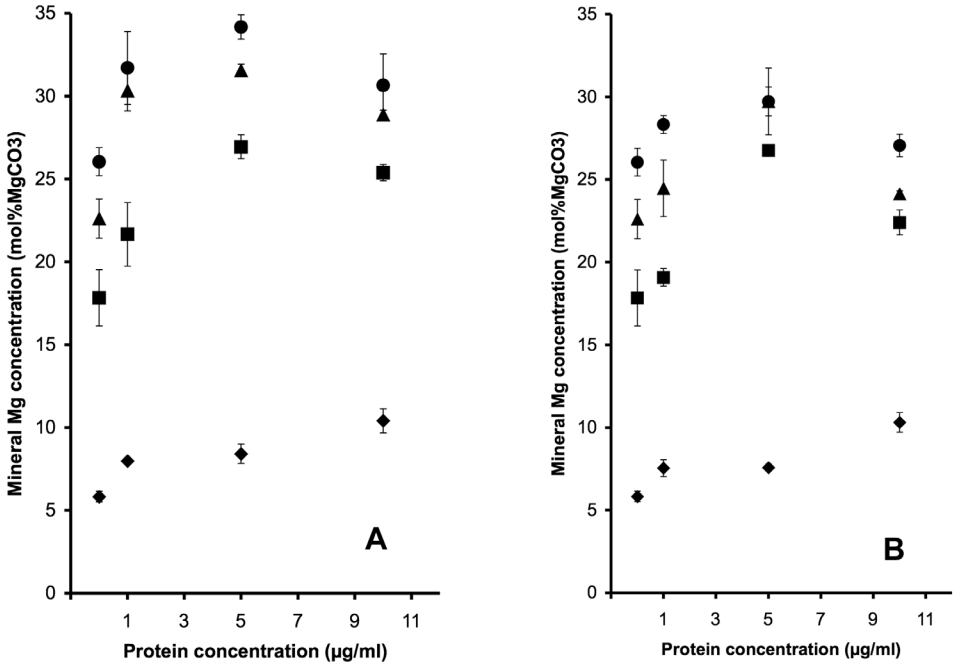

Figure 2. Mean magnesium concentration (±standard deviation, $n = 4$) in minerals precipitated in vitro in the presence of (a) test and (b) spine protein extracts, according to protein concentration and solution Mg/Ca (solid diamond, 1:1 mol/mol; solid square, 3:1 mol/mol; solid triangle, 4:1 mol/mol; solid circle, 5:1 mol/mol).

With low protein concentration and $Mg/Ca_{solution}$, elongated crystals, smaller (ca. from 60 to 80 μm) but more abundant than those formed in the absence of proteins, were observed (Figures 5a and 6a). Higher protein concentrations at low $Mg/Ca_{solution}$ induced formation of dumbbell-shaped minerals, with angular extremities at 5 μg/mL concentrations (Figures 5b and 6b) and rounded extremities at 10 μg/mL (Figures 5c and 6c). At 3:1, 4:1, and 5:1 $Mg/Ca_{solution}$ ratios, we could observe a dense cover of spherical particles of 1 μm diameter on cracked film (Figures 5d and 5e). These particles formed chains resulting in small to large aggregates, without any correlation between the size of the aggregate and the $Mg/Ca_{solution}$, nature, or concentration of the matrix (Figures 5f and 6d).

4. Discussion

[23] The magnesium concentration in the deposited minerals increased with the Mg/Ca ratio of the solution, as reported in previous studies [*Falini et al.*, 1994; *Han et al.*, 2005; *Cheng et al.*, 2007]. It reached values similar to those measured by other authors, including in the absence of organic additive [*Raz et al.*, 2000; *Loste et al.*, 2003]. This factor had a predominant effect on magnesium incorporation in comparison with the nature and concentration of soluble organic matrix (SOM). Therefore, a biological control of the precipitation solution could be sufficient to reach elevated magnesium concentrations reported in biogenic calcites.

[24] Most cases of biologically controlled biomineralizations occur in a compartment isolated from the external environment and delimited by a biological membrane (vacuole, intercellular space) [*Lowenstam and Weiner*, 1989;

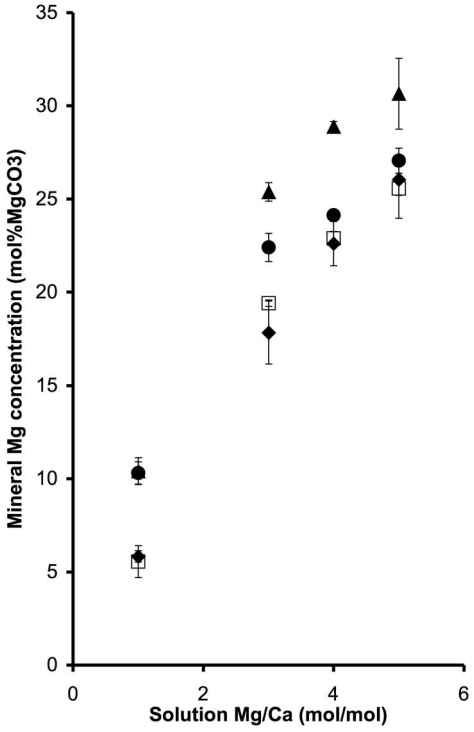

Figure 3. Magnesium concentration in minerals precipitated in vitro in the absence of protein (solid diamond) and in the presence of 10 μg/mL BSA (square), test (solid triangle), or spine (solid circle) protein extracts, according to the solution Mg/Ca.

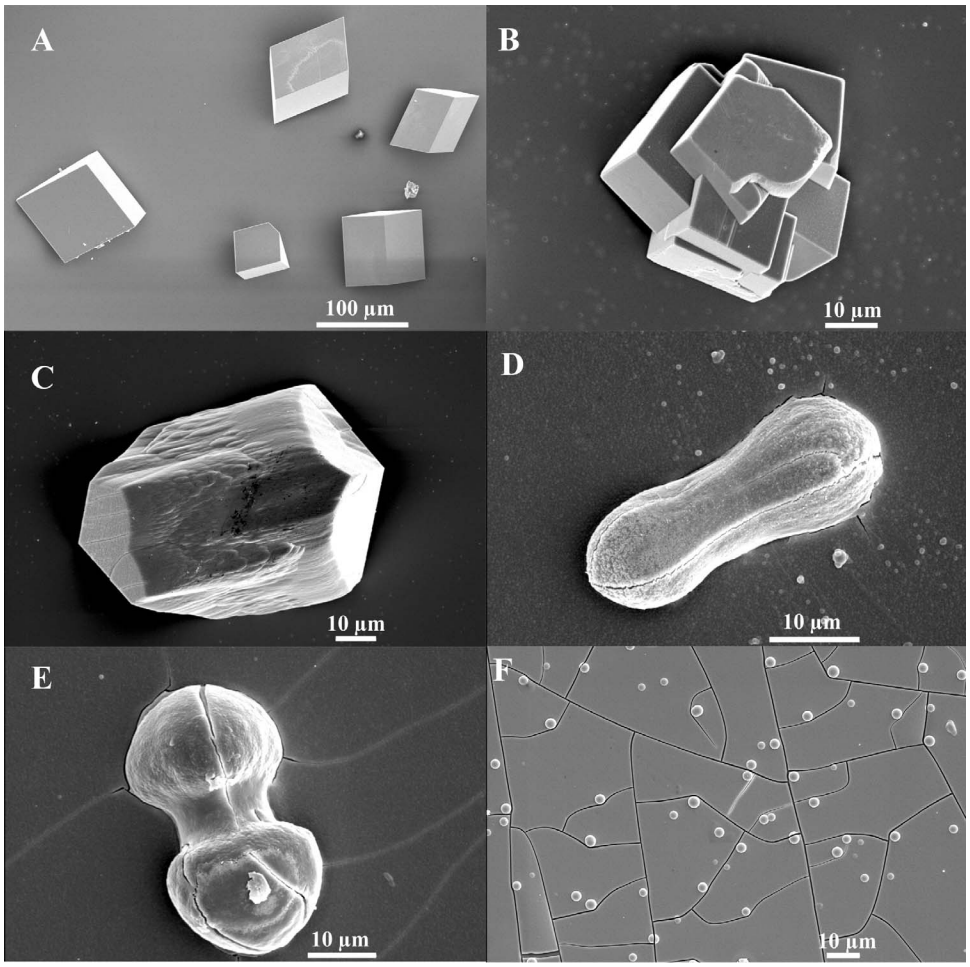

Figure 4. Scanning electron micrographs of precipitated mineral grown (a) in the absence of magnesium and organic additive, (b) in the absence of magnesium with 10 μg/mL test protein extract, in the absence of organic additive in (c) 1:1, (d and e) 3:1, and (f) 4:1 Mg/Ca solutions.

Simkiss and Wilbur, 1989]. The ionic composition in the mineralization compartment can therefore be strictly biologically controlled by the exchanges through this membrane. Seawater Mg/Ca ratio has been shown to influence the skeletal Mg/Ca ratio of calcifying organisms [*Lorens and Bender*, 1980; *Ries*, 2004] to a certain degree, and it must therefore exert some influence on the Mg/Ca of the solution in the calcification site. This dependence on seawater Mg/Ca indicates that some nonselective transport mechanisms, such as diffusion, are probably also involved.

[25] However, the Mg/Ca ratio of the in vitro inorganically precipitated minerals far exceeded values measured in both past and present sea urchin skeletons. Using the algorithms of *Ries* [2004], hypothetical skeletal Mg/Ca ratios of sea urchins grown in different $Mg/Ca_{solution}$ were calculated (Figure 1). All calculated values were much below the ratio measured for in vitro precipitated minerals. Therefore, magnesium-specific transport mechanisms should be involved in the determination of the Mg/Ca ratio in the calcifying space, probably lowering the magnesium concentration. In this context, it should be noticed that in vitro deposits were grown from pure solutions of calcium, magnesium, carbonates, and chloride, whereas in nature ions like sodium are very probably present and also influence the properties of the precipitated biogenic calcites [*Morse and Mackenzie*, 1990].

[26] We recorded a significant enhancement of magnesium incorporation in the presence of SOM extracted from the sea urchin skeleton in comparison to precipitation experiments performed in the absence of organic additive. This effect was not observed with bovine serum albumin, which indicates that the SOM effect was specific. Moreover, the enhancement of magnesium incorporation was more pronounced with SOM extracted from the test than with those extracted from the spines. With the Mg/Ca ratio of *P. lividus* test being higher than the Mg/Ca ratio of the spines, this result further supports the hypothesis that SOM has a specific effect on magnesium incorporation. This effect is probably modulated by organic matrix composition and/or concentration.

[27] Indeed, amino acid compositions of sea urchin test and spine SOM were relatively different: the test SOM contained more aspartate and glycine and less proline than the spines. High concentrations of glycine had already been reported in the skeletal organic matrix of seastars [*Gayathri et al.*, 2007; P. Dubois, unpublished results]. *Aizenberg*

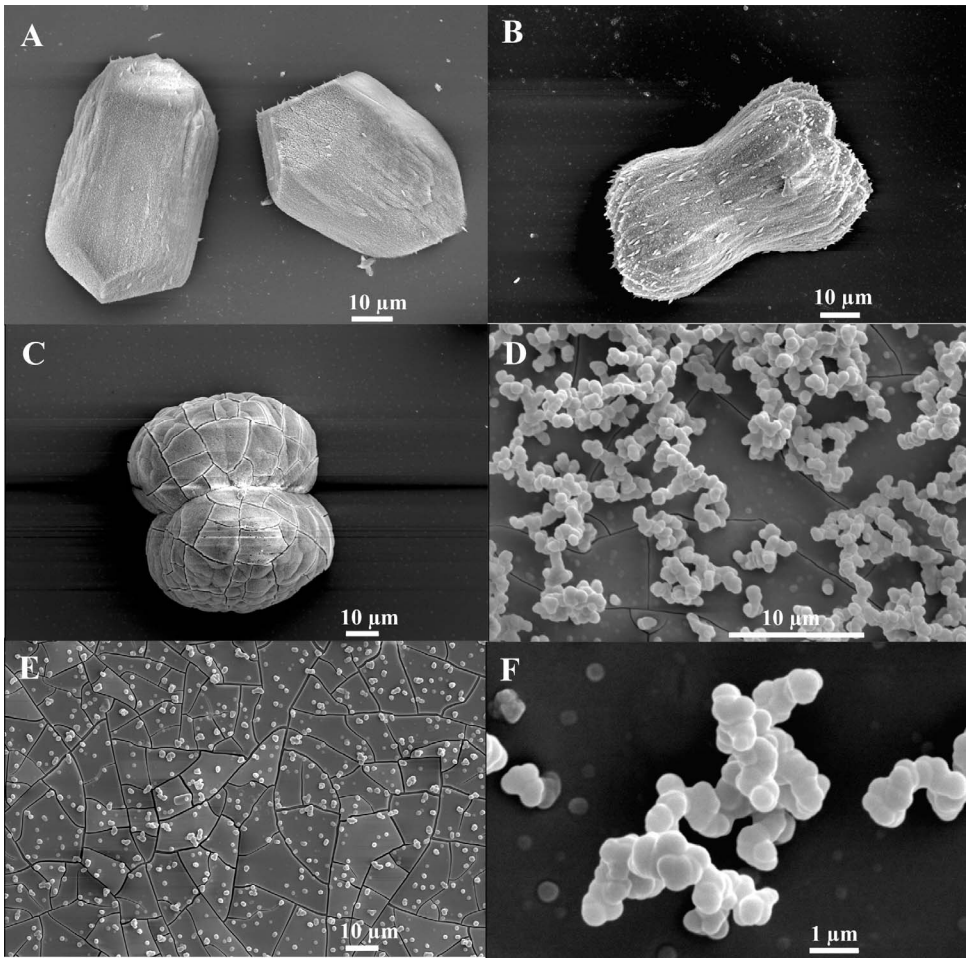

Figure 5. Scanning electron micrographs of precipitated mineral grown in the presence of spine protein extract: (a) 1:1 Mg/Ca solution, 1 μg/mL protein; (b) 1:1 Mg/Ca solution, 5 μg/mL protein; (c) 1:1 Mg/Ca solution, 10 μg/mL protein; (d) 3:1 Mg/Ca solution, 5 μg/mL protein; (e) 4:1 Mg/Ca solution, 1 μg/mL protein; (f) 4:1 Mg/Ca solution, 5 μg/mL protein experiments.

et al. [1996] showed that this amino acid was more abundant in the amorphous phase than in the crystalline phase of the spicules of the sponge *Clathrina* sp., and they suggested its involvement in the stabilization of such phases. The relative abundance of aspartic acid was also higher in the test than in the spine SOM. Aspartic-rich proteins, which possess a domain that possibly binds magnesium ions [*Gotliv et al.*, 2005], were suggested to play an important role in the incorporation of this cation. Moreover, these proteins were shown to stabilize amorphous calcium carbonate (ACC) [*Politi et al.*, 2007] that affected the magnesium signature of calcite [*Loste et al.*, 2003]. In sea urchins, the saturation of such proteins has been suggested to limit the increase of magnesium incorporation with increasing temperature [*Hermans et al.*, 2010].

[28] A quadratic modulation of the magnesium incorporation in the precipitated minerals by the protein concentration was observed at $Mg/Ca_{solution} \geq 3$. The magnesium incorporation was higher at a protein concentration of 5 μg/mL than at 1 and 10 μg/mL. *Wang et al.* [2009] observed a similar effect with aspartic and glutamic acids. They suggested that this was due to the saturation of either ACC, aspartic and glutamic acids, or both in their ability to interact with calcium.

[29] The observed enhancement of magnesium incorporation in the presence of genuine organic matrix compared to controls without matrix was maximal in the precipitation experiments performed at a 3:1 $Mg/Ca_{solution}$ with a protein concentration of 5 μg/mL, for both matrix extracts. In these treatments, precipitated minerals were richer by 9 mol % $MgCO_3$ than those precipitated in the absence of additive. Such an important effect of the SOM on magnesium incorporation questions the use of skeletal magnesium concentration as a record of the temperature of formation. It probably explains why the relations between skeletal magnesium concentration and environmental temperature are poorly constrained in echinoderms [*Weber*, 1969, 1973; *Dickson*, 2002], making this phylum a rather poor candidate for paleoreconstructions. Furthermore, it raises the question of whether the composition of the SOM may change according to physiological or environmental factors, further degrading the relation. Further studies of variations in magnesium incorporation linked to the SOM, in taxa regularly used as environmental archives, would be worthwhile.

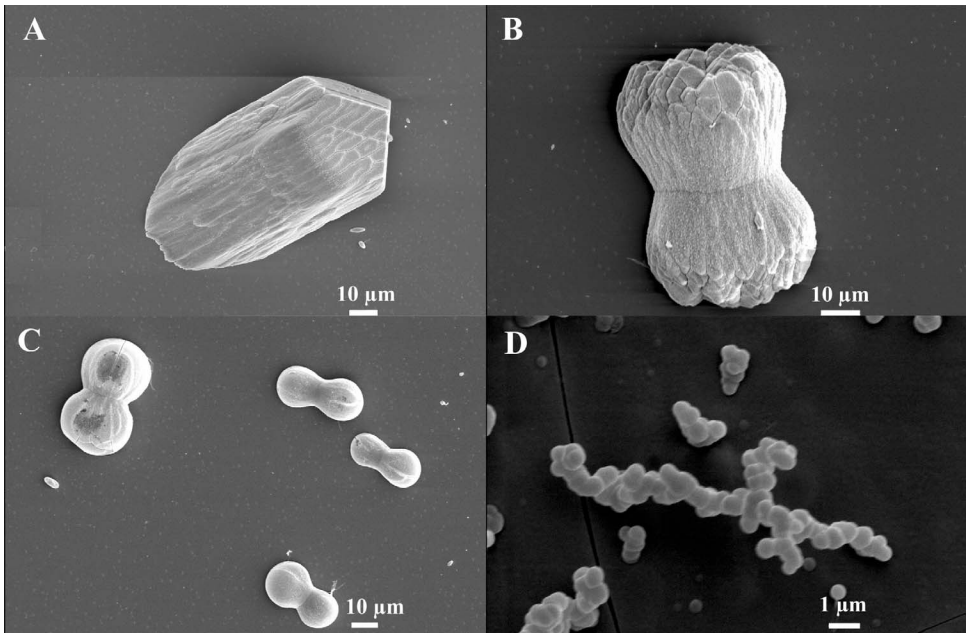

Figure 6. Scanning electron micrographs of precipitated mineral grown in the presence of test protein extract: (a) 1:1 Mg/Ca solution, 1 μg/mL protein; (b) 1:1 Mg/Ca solution, 5 μg/mL protein; (c) 1:1 Mg/Ca solution, 10 μg/mL protein; (d) 4:1 Mg/Ca solution, 10 μg/mL protein experiments.

[30] We do notice that our results differed from those of *Raz et al.* [2003], who did not observe an increase of magnesium incorporation in minerals precipitated in the presence of organic matrix macromolecules extracted from sea urchin larval spicules. We suggest that this absence of effect could be due to either the low $Mg/Ca_{solution}$ (2:1) used by these authors, or to the lyophilization to dryness of the SOM which could have affected the conformation of these macromolecules. The differences observed from the results of *Falini et al.* [1994] are probably linked to the analytical method, X-ray diffraction, that did not take into account magnesium in the amorphous phase.

[31] The morphology of in vitro precipitated minerals was relatively similar to that described in the literature. The magnesium-induced curving of crystal edges was already reported by *Meldrum and Hyde* [2001]. The elongation of crystal along the *c* axis in the presence of organic additives was observed by *Albeck et al.* [1993, 1996a] and *Meldrum and Hyde* [2001]. The dumbbell-shaped minerals have been characterized as magnesium calcite [*Raz et al.*, 2000; *Meldrum and Hyde*, 2001; *Gayathri et al.*, 2007]. Peanut-like deposits were reported by *Loste et al.* [2003]. Spherical particles similar to those observed in this study at high $Mg/Ca_{solution}$ were reported in previous studies [*Han et al.*, 2005; *Kwak et al.*, 2005; *Cheng et al.*, 2007] and characterized as a mixture of calcite and ACC with high magnesium concentration. *Kwak et al.* [2005] highlighted the importance of the crystallization pathway in impurity entrapment into calcite [*Kwak et al.*, 2005]. Mineralization through an ACC transient phase is suggested to be an essential step in the formation of high magnesium calcites [*Raz et al.*, 2000; *Loste et al.*, 2003; *Cheng et al.*, 2007] and has been shown to be favored in magnesium-rich solutions [*Kwak et al.*, 2005]. ACC has been suggested to be stabilized by high concentrations of magnesium in the solution [*Raz et al.*, 2000; *Loste et al.*, 2003] or in the mineral [*Politi et al.*, 2010]. Moreover, the stabilization of ACC has been demonstrated to be also partially completed by the organic matrix [*Raz et al.*, 2000, 2003; *Aizenberg et al.*, 2002; *Politi et al.*, 2007]. Once formed, this amorphous phase favors the incorporation of elevated quantities of magnesium, and these high concentrations are conserved in the crystal after crystallization and co-occurring water expulsion [*Loste et al.*, 2003]. *Raz et al.* [2000] suggested that the incorporation of high magnesium levels results from a combined effect of an elevated Mg/Ca ratio in the precipitation compartment and the involvement of an organic matrix inducing the formation and stabilization of an ACC. Indeed, *Wang et al.* [2009] showed that carboxylic acids, including aspartic and glutamic acids, do increase the incorporation of magnesium in ACC. Our observations suggest a possible link between the relative abundance of aspartic acid rich proteins in the organic matrix and the magnesium incorporation, which probably proceed through ACC stabilization by the Asp-rich proteins. It is noteworthy that *Aizenberg et al.* [2002] reported that Glu-rich and not Asp-rich proteins are linked to ACC parts of sponge and ascidian spicules. However, *Wang et al.* [2009] suggested that not only the primary structure of proteins is important but also their stereochemistry is important. Alternatively, the effect of Asp-rich proteins on magnesium incorporation could be linked to kinetic factors [*Wang et al.*, 2009].

[32] In conclusion, we showed that solution Mg/Ca ratio is the main factor affecting magnesium incorporation for in vitro precipitated calcium carbonate deposits. Organic matrix macromolecules induced an enhancement of magnesium incorporation in the mineral. This effect has been shown to be specific and more pronounced for SOM extracted from a magnesium-richer skeletal element. We suggest that the origin of this enhancement arises from the

combined effects of high Mg/Ca ratio in solution and the presence of organic matrix, both inducing and stabilizing a magnesium-rich amorphous phase. The involvement of the organic matrix in this process can explain the observation that sympatric organisms or even different skeletal elements of the same individual present different magnesium concentrations in the mineralized skeleton.

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