Interactions of Various Skeletal Intracrystalline Components with Calcite Crystals

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Abstract: The calcitic skeletal elements of many organisms contain small amounts of proteins and glycoproteins which are incorporated within single crystals of calcite. Extraction and partial purification of the intracrystalline macromolecules allowed the identification of their interactions with synthetic calcite crystals. Specific interactions were inferred from induced morphological modifications associated with the development of new faces on calcite crystals grown in the presence of these macromolecules in solution. Partially purified macromolecules extracted from within sea urchin spines interacted only with faces roughly parallel to the *c* crystallographic axis of calcite, producing well-developed {01*l*} faces. Macromolecules extracted from within mollusk shell prisms separated into two fractions each having a characteristic amino acid composition and displayed distinct specific interactions with growing calcite crystals: A highly acidic fraction interacts with the {001} set of faces, while a different fraction, which is only moderately acidic and has an amino acid composition similar to that of the sea urchin spines, interacts specifically with the {01*l*} set of faces. Magnesium, a major ionic component of the sea urchin spines, causes the development of the {011} set of crystal faces. The existing knowledge on the direction of intercalation of the macromolecules in the biogenic crystals agrees with the interacting planes in the synthetic crystals, suggesting the relevance of our *in vitro* assay to the system *in vivo*. We suggest that each separate mode of interaction may infer a specific role in the regulation of biological crystal growth.

Introduction

Many organisms form skeletal elements by precipitating calcium carbonate crystals. The site of nucleation, crystal orientation, crystal morphology, the polymorphic phase, and even mechanical properties may all be controlled by the biological environment in which the crystals form.[1]

One common method used by organisms for controlling crystal formation is to grow the crystal in a preformed space composed of either macromolecules or lipid bilayers. In this way both the solution composition and the final shape, size, and orientation of the crystal can be locally modulated.[2] Recently, an additional strategy has been identified, namely that macromolecules interact from the crystallizing solution with specific planes of the growing crystal. In so doing they retard growth in the direction perpendicular to the plane and, in effect, control crystal morphology. After being adsorbed on the crystal surface, they are overgrown and are consequently occluded within the crystal, where they may also affect its mechanical properties and texture.[3]

Following stringent removal of all organic material on the crystal surface and by dissolution of the crystal elements, a suite of intracrystalline macromolecules and various ions over and above calcium and carbonate are released into solution.[4] These macromolecules have been partially characterized in certain brachiopods,[5] cnidaria,[6] mollusks,[4,7] and echinoderms.[8,9] The different ensembles of intracrystalline macromolecules share some common features. They all contain a large water-soluble protein component which is characterized by a high content of Asx, Glx, and Gly. In many cases these proteins have been shown to be glycosylated.[4,8] Some skeletal elements contain a relatively large amount of magnesium ions.[10] The concentrations and properties of these intracrystalline components vary between skeletal elements, suggesting that organisms employ different means of controlling crystal growth from solution.

The ways in which intracrystalline macromolecules influence crystal growth and contribute to the function of the skeletal element have in part been inferred from the effect they have on crystal texture. Synchrotron X-ray studies of synthetic and biologically produced calcite crystals demonstrate that the average distance between lattice imperfections in a given direction (coherence length) and the degree of misalignment of coherent blocks are influenced by occluded macromolecules.[11] Complementary information can be obtained from *in vitro* experiments where the ions and/or macromolecules are added to the crystallizing solution and the manner in which they influence nucleation and growth is monitored indirectly from change in the bulk solution properties.[12] An alternative *in vitro* experimental design to address this question is to monitor changes in crystal morphology that result from the presence of an additive in solution.[13] Morphological changes result from specific binding

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(1) (a) Lowenstam, H. A.; Weiner, S. *On Biomineralization*; Oxford University Press: New York, Oxford, 1989. (b) Simkiss, K.; Wilbur, K. M. *Biomineralization, Cell Biology and Mineral Deposition*; Academic Press, Inc.: San Diego, New York, 1989.

(2) Simkiss, K. In *Biomineralization in Lower Plants and Animals*; Leadbeater, B. S. C., Riding, R., Eds.; Clarendon Press: Oxford, U.K., 1986; pp 19–37.

(3) Addadi, L.; Weiner, S. *Angew. Chem.* **1992**, *31*, 153–169.

(4) Crenshaw, M. A. *Biomineralization* **1972**, *6*, 6–11.

(5) (a) Curry, G. B.; Cusack, M.; Walton, D.; Endo, K.; Clegg, H.; Abbot, G.; Armstrong, H. *Philos. Trans. R. Soc. London*, B 1991, *333*, 359–366. (b) Curry, G. B.; Cusack, M.; Endo, K.; Walton, D.; Quinn, R. In *Mechanisms and Phylogeny of Mineralization in Biological Systems*; Suga, S., Nakahara, H., Eds.; Springer-Verlag: Tokyo, 1991; pp 35–39.

(6) Kingsley, R. J.; Watabe, N. *Comp. Biochem. Physiol., B* **1983**, *76*, 443–447.

(7) Addadi, L.; Berman, A.; Weiner, S. In *Mechanisms and Phylogeny of Mineralization in Biological Systems*; Suga, S., Nakahara, H., Eds.; Springer-Verlag: Tokyo, 1991; pp 29–33.

(8) Benson, S.; Benson, N. C.; Wilt, F. *J. Cell Biol.* **1986**, *102*, 1878–1886.

(9) Weiner, S. *J. Exp. Zool.* **1985**, *234*, 7–15.

(10) (a) Chave, K. E. *J. Geol.* **1954**, *62*, 266–283. (b) Dubois, Ph. In *Echinoderm Research*; De Ridder, C., Dubois, Ph., Lahaye, M. Ch., Jangoux, M., Eds.; Balkema: Rotterdam, The Netherlands, 1990; pp 17–22.

(11) (a) Berman, A.; Addadi, L.; Kvick, A.; Leiserowitz, L.; Nelson, M.; Weiner, S. *Science* **1990**, *250*, 664–667. (b) Berman, A.; Hanson, J.; Leiserowitz, L.; Koetzle, T. F.; Weiner, S.; Addadi, L. *Science* **1993**, *259*, 776–779.

(12) (a) Wheeler, A. P.; Sikes, C. S. In *Biomineralization, Chemical and Biochemical Perspectives*; Mann, S., Webb, J., Williams, R. J. P., Eds.; VCH Verlagsgesellschaft: Weinheim, Germany, 1989; pp 95–131. (b) Nancollas, G. H. In *Biomineralization, Chemical and Biochemical Perspectives*; Mann, S., Webb, J., Williams, R. J. P., Eds.; VCH Verlagsgesellschaft: Weinheim, Germany, 1989; pp 157–187.

(13) Addadi, L.; Berkovitch-Yellin, Z.; Weissbuch, I.; Van Mil, J.; Shimon, L. J. W.; Lahav, M.; Leiserowitz, L. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 466–485.

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of the additive to a particular crystal face, which in turn retards crystal growth in the direction perpendicular to that face. This latter type of experiment has been used to assay whole assemblages of intracrystalline macromolecules.[14] In the case of the sea urchin macromolecules, a set of rough faces more or less parallel to the *c* axis developed.[7,15]

This study focuses on the ways in which various occluded macromolecules and magnesium ions interact from solution with growing calcite crystals. The approach used is to isolate and purify different constituents from the biogenic crystal and then use the *in vitro* crystal habit assay to determine how each component interacts with forming calcite crystals. We compare and contrast the constituents of two quite different biogenic calcites: prisms from the outer layer of a mollusk shell that are formed in an extracellular macromolecular matrix and spines from a sea urchin that grow in lipid membrane vesicles.[1] We also address the role magnesium ions may play in the mineralization process.

Experimental Section

Skeletal Element Preparation. Shells of the bivalve mollusk *Atrina rigida* (North Carolina, USA) were collected fresh, cleaned, and stored dry. The calcitic prismatic layer was mechanically separated from the inner nacreous layer, and the former was treated with 12% NaOCl solution with constant stirring for 4 days. The crystal suspension was sonicated for 10 min, and the NaOCl was removed by decantation. The crystals were then extensively washed with double-distilled water (DDW).

Adult specimens of the echinoid *Paracentrotus lividus* were collected alive at Caesarea, Israel. The spines were removed and treated with a 2.5% solution of NaOCl overnight on a rocking table to remove extraskeletal organic residue. The spines were then extensively washed with DDW and stored dry at room temperature.

The crystalline skeletal elements were examined in a Jeol 6400 scanning electron microscope (SEM), to verify surface cleanliness and, in the case of the mollusk shell, to verify that the single-crystalline elements were completely disaggregated.

Recrystallization Experiments. Samples of clean crushed skeletal elements were suspended in DDW (1 mg of mineral/10 mL of water). Stoichiometric amounts of concentrated HCl (2 mol of HCl/1 mol of mineral) were added dropwise over several hours with a microcapillary pipette. The pH of the final solution was 6.7. The solution was centrifuged to remove insoluble material, and the supernatant was diluted with DDW to yield a 100 mM solution with respect to calcium. This solution was introduced directly into a well of a Nunc multidish used for cell culture (well diameters 1.5 cm) which contained a glass coverslip. Calcite crystals were grown for 3 days in a closed desiccator which contained vials of ammonium carbonate powder.[14] Following crystallization, the coverslip with the crystals was rinsed with DDW and glued to a SEM stub.

Extraction of Intraskeletal Macromolecules. A. EDTA Extraction. The clean, dry single crystals were crushed and decalcified by dialysis (Spectrapor 3 tubing with a molecular weight cutoff of 3500 D) against a 0.5 M solution of EDTA (ethylenediaminetetraacetic acid) adjusted to pH 8.0 with NaOH and buffered with 0.05 M Tris base (Tris-(hydroxymethyl)aminomethane). The solution contained 0.01% (wt/vol) sodium azide.[9,16] After complete decalcification, the contents of the dialysis bag were dialyzed exhaustively against four changes of DDW over 2 days, lyophilized, and redialyzed against another two changes of DDW. The insoluble material was removed by centrifugation at 3000*g* for 10 min. The soluble material was lyophilized and stored at −4 °C for analysis and use in the growth experiments.

B. HCl Extraction. Samples of crushed, clean, dry single crystals were suspended in DDW, and stoichiometric amounts of concentrated HCl were added dropwise over several hours. The final pH of the solution was 6.7. This solution was extensively dialyzed against DDW, and the soluble and insoluble materials were treated as described for the EDTA-extracted material.

Amino Acid Analyses. Aliquots of the protein solution were hydrolyzed under vacuum in 0.3 mL of 6 N HCl at 112 °C for 24 h after flushing twice with nitrogen. The hydrolysates were analyzed on a Dionex BIOLC amino acid analyzer.

Anion-Exchange Chromatography. Aliquots (10 mg) of water-soluble protein were dissolved in 1 mL of DDW and loaded on a DE52 ion-exchange column (34 × 1.2 cm) (Whatman, Maidstone, Great Britain) buffered with 0.05 M Tris base, pH 8.2.[16] Either a linear NaCl gradient or a two-step gradient (0.35 M NaCl, 1.00 M NaCl) in starting buffer was used. The column was flushed at the end of the run with a 2 M NaCl solution. Two-milliliter fractions were collected, and their absorbances at 280 and 230 nm were measured. The NaCl concentration of the fractions was determined by measuring conductivity. The fractions were then pooled according to their absorbance. The pooled fractions were extensively dialyzed as described for the EDTA-extracted macromolecules.

High-Performance Liquid Chromatography. High-performance liquid chromatography was carried out with a Waters UV detector (Model 440, wavelengths 254 and 230 nm), a Dionex gradient pump, and Dionex injector with a 50-μL sample loop. An anion-exchange Mono Q column (Pharmacia, HR 5/5) was used. The column was equilibrated with a buffer solution of 0.05 M Tris base, pH 8.2. The buffer solution and a 1 M NaCl solution in buffer were degassed under reduced pressure in an ultrasonic bath for 10 min. A 35-min, two-step gradient was initiated immediately after injection of 50 μL of sample (0.5 M NaCl, 1.0 M NaCl). The proteins were eluted at room temperature at a flow rate of 0.5 mL/min. After the two gradients were complete, a reverse gradient was applied to return the column to the initial conditions. The fractions were then pooled and treated as in the procedure described for ion-exchange chromatography.

Crystal Growth Experiments. Synthetic calcite crystals were grown in Nunc multidishes with well diameters of 1.5 cm in which clean glass cover slips of 1.3-cm diameter were placed. A total volume of 0.75 mL of 7.5 mM calcium chloride solution was introduced to each well. The wells were covered with aluminum foil and punctured by a needle. Calcite crystals were grown for 3 days by slow diffusion of ammonium carbonate in a closed desiccator.[14] When the effect of protein was studied, aliquots of concentrated protein solution were added to the above calcium chloride solution. The effect of magnesium was studied by adding various amounts of magnesium chloride to the calcium chloride solutions. In each crystallization experiment, controls which contained the pure calcium chloride solution without additives were analyzed. The crystals grown in the absence of protein were always perfect rhombohedra.

The glass coverslips covered with calcite crystals were lightly rinsed with DDW, dried, and glued to SEM stubs. After gold coating, the affected crystals were observed in the SEM. The calcite crystals grown in the presence of the sea urchin spine protein or magnesium ions expressed new {0*kl*} faces. In order to identify these new faces, the crystals were viewed with their {0*kl*} and corresponding {104} faces both edge-on. In this position the *c* crystallographic axis lies in the plane of the picture, allowing the measurement of the α angle, which is the angle between the *c* axis and the unknown {0*kl*} face.[17] This angle unequivocally identifies the Miller indices of the face.[18] The sign of *k* and *l* is defined by the angle between the new face and the {104} face both in the edge-on position. When *k* and *l* are of the same sign, the new face will form with the (104) face an angle of $(135° - \alpha)$ while a face indexed by *k* and *l* having opposite signs will form an angle of $(135 + \alpha)$ (Figures 1a and 2).

Results

The strategy we have adopted in this study is to first examine the effect of the entire ensemble of intracrystalline components, both ions and macromolecules, acting together on growing calcite crystals. This was performed by first dissolving the biogenic elements and then recrystallizing new calcite crystals *in vitro* from this solution. Next the effect of only the total extracted macromolecular assemblages on growing calcite crystals was determined. The assemblage of macromolecules was then further fractionated, and the manner in which each fraction interacts with growing calcite crystals was examined. Finally, the effect of magnesium, which is a major ionic component of the biogenic

(14) Addadi, L.; Weiner, S. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 4110–4114.
(15) Berman, A.; Addadi, L.; Weiner, S. *Nature* **1988**, *331*, 546–548.
(16) Weiner, S. *Calcif. Tissue Int.* **1979**, *29*, 163–167.
(17) Crystal faces are denoted by a set of Miller indices *h*, *k*, *l*, that defines their orientation relative to the crystal axis. {*h*,*k*,*l*} denotes the family of symmetry related faces (*h*,*k*,*l*). For calcite, for example {104} = (104), ($\bar{1}$14), (0$\bar{1}$4), ($\bar{1}$04), ($\bar{1}\bar{1}$4), (01$\bar{4}$).
(18) Aizenberg, J.; Albeck, S.; Weiner, S.; Addadi, L. *Proc. Natl. Acad. Sci. U.S.A.* Submitted for publication.

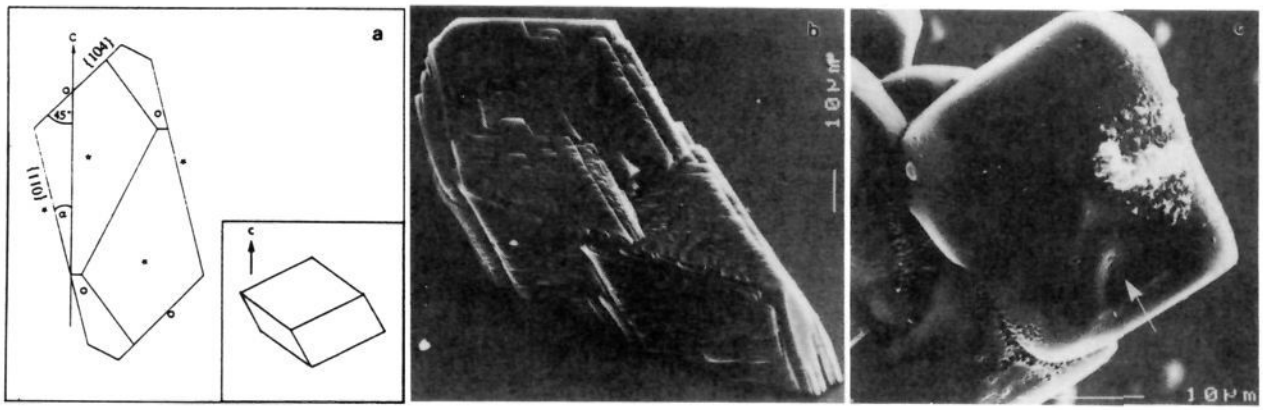

Figure 1. (a) Computer simulation of the morphology of a calcite crystal expressing {011} faces.[17] One of the {104} faces and the corresponding {011} face are viewed edge-on in a position which allows the determination of the angle α ($\alpha = 14°$ for the {011} set of faces). Faces marked by O are members of the {104} set of faces, while faces marked with * are members of the {011} set. Insert: Computer simulation of the morphology of a pure calcite crystal. All the developed faces are {104}. (b) Synthetic calcite crystals obtained from the recrystallization of a solution of dissolved sea urchin spines. Note the development of large {011} faces and very small remnants of the cleavage rhombohedral {104} faces as in part a. (c) Synthetic calcite crystals obtained from the recrystallization of a solution of dissolved mollusk prisms. Note the small size of the crystal, its smooth glassy appearance, and the development of a small (001) face (marked by an arrow).

Table I. Amino Acid Compositions of Soluble Macromolecules Extracted from within Sea Urchin Spines and Mollusk Prisms Using EDTA or HCl, and the Protein Yield from the Two Skeletons Using Both Extraction Methods

	sea urchin spines		mollusk prisms	
	EDTA	HCl	EDTA	HCl
[protein][a]	0.009	0.015	0.13	0.23
Asx	10.0	12.1	53.6	49.5
Thr	5.0	4.6	1.8	2.2
Ser	4.6	3.6	3.8	3.6
Glx	10.9	12.9	20.5	25.8
Pro	10.3	10.1		
Gly	15.4	19.1	4.0	3.6
Ala	11.5	9.9	9.2	8.3
Cys	0.7	0.2	0.4	0.2
Val	5.8	4.3	2.1	1.9
Met	4.3	4.9	0.2	0.3
Ile	3.5	3.0	0.5	0.5
Leu	5.6	3.7	1.1	1.1
Tyr	1.6	2.7	0.4	0.6
Phe	3.8	3.6	0.5	0.5
Lys	1.6	1.2	1.0	1.0
His	2.0	1.2	0.2	0.2
Arg	3.4	2.9	0.7	0.7

[a] Protein concentration expressed in terms of mole % amino acids/mole mineral.

crystals, was studied separately, in the absence and presence of the proteinacious components.

Recrystallization Experiments. Calcite crystals were induced to form *de novo* from a solution of dissolved biogenic crystals. In this way the newly formed crystals can interact during growth with all the biogenic intracrystalline components released into solution in their original concentrations and relative proportions. Each of these components may interact specifically with a certain face or nonspecifically with all surfaces of the growing crystal. When more than one component is present in solution, each component may interact differently, resulting in a combined effect.

The mollusk prisms contain about 0.2 mole % proteins which are very rich in aspartic acid and glutamic acid.[16] The sea urchin spines contain 0.015 mole % protein, and these are much less acidic than those extracted from the mollusk (Table I). Another important difference between the two biogenic calcite crystals is the magnesium ion content; the sea urchin contains 7.5 mole % magnesium carbonate, while magnesium is almost undetectable in the mollusk prisms.[11b]

Recrystallization of a solution of dissolved sea urchin spines yielded crystals with large, well-developed {011} faces (Figure 1a,b). The recrystallization of a solution of dissolved mollusk prisms produced very few small, smooth, and rounded crystals (Figure 1c). In the latter case we could sometimes detect some specific inhibition of growth in the direction parallel to the *c* axis, on the basis of the development of small {001} faces. These observations indicate that the intracrystalline components of the sea urchin spine and the mollusk prism interact quite differently with growing calcite crystals. In order to better understand the different interactions involved in the two recrystallization experiments, it is necessary to examine the ways in which each of the components interacts separately with growing calcite crystals.

Total Intracrystalline Macromolecular Assemblages. Several technical difficulties are associated with the extraction of the macromolecules from within the calcitic skeletal elements. EDTA, a strong calcium ion chelator, has the advantage of dissolving the mineral at neutral pH and hence under conditions which are most likely to protect the proteins. However, traces of EDTA, which are extremely difficult to remove,[19] may interact independently with growing calcite crystals. Moreover, EDTA presumably remains bound in small amounts to the strongly ionic macromolecules and may influence their charge, conformation, and state of aggregation. This could well affect the manner in which the proteins interact with crystals. Dissolution of the biogenic calcite with HCl is an alternative approach. It has been reported[20] that the amino acid composition of the matrix varied little when shells were decalcified by EDTA or dilute acids. Furthermore, a high capacity of calcium binding could still be detected in a matrix that was decalcified by HCl.[21] However, there is always the risk that the low-pH conditions can somehow affect the protein. We have used both methods of extraction, followed by extensive dialysis of the solution to remove excess ions and low molecular weight components. Note that the protein yield is higher using HCl dissolution as compared to EDTA (Table I). This may be due to aggregation of certain low molecular weight components, induced by the acidic environment, which are lost during dialysis when extracted with EDTA. Another possibility is that, during the slow decalcification by EDTA, protein may be lost by breakdown and leakage through the dialysis membrane or that certain components may remain bound via EDTA to the dialysis membrane.[22] Using the sea urchin spines, we have noticed that the EDTA extraction method removes the

(19) Wheeler, A. P.; Rusenko, K. W.; George, J. W.; Sikes, C. S. *Comp. Biochem. Physiol.* **1987**, *87B*, 953–960.

(20) Kasai, H.; Ohta, N. In *Professor Masae Omori Memorial Volume Publication Committee*; Habe, T., Omori, M., Eds.; Niigata University Press: Niigata, 1981; pp 101–106.

(21) Samata, T. *Venus* **1988**, *47* (2), 127–140.

Table II. Amino Acid Compositions of Fractions from the Total Extract of Mollusk-Shell Prisms Using EDTA or HCl, after Fractionation on a DEAE Ion-Exchange Column[a]

	EDTA			HCl			
	PE1	PE2	PE3	PH1	PH2	PH3	PH4
[NaCl][b]	0–0.2	0.2–0.3	0.3–1.0	0–0.15	0.15–0.39	0.39–0.5	0.5–1.0
[protein][c]	1.4	1	97.6	1	6	82	11
Asx	10.3	12.1	58.9	9.9	12.8	60.0	25.2
Thr	8.0	7.1	1.1	6.9	7.5	1.1	1.7
Ser	10.8	8.8	3.2	12.3	6.7	2.9	2.1
Glx	18.4	17.0	22.1	17.6	29.0	22.5	58.1
Pro	6.4	4.0		8.7	4.5		
Gly	10.6	10.1	2.9	13.8	4.0	3.0	2.0
Ala	10.4	13.6	8.1	8.2	13.2	8.2	6.6
Cys	0.7	0.8			1.6		0.3
Val	4.7	4.9	1.6	4.1	4.2	1.6	1.2
Met	1.2	1.3	0.1	1.5	1.3		0.1
Ile	2.5	3.1	0.2	2.6	2.0	0.1	0.3
Leu	4.0	6.7	0.8	3.4	3.5	0.4	0.6
Tyr	2.5	0.9	0.2	2.1	1.4		0.4
Phe	1.7	2.3	0.1	1.4	2.4		0.3
Lys	4.2	4.1	0.6	3.5	3.4	0.1	0.6
His	1.6	0.7		2.0	0.6		0.1
Arg	2.0	2.5	0.1	2.0	1.9	0.1	0.4

[a] Amino acid compositions are expressed in terms of mole %. [b] Elution position of the fraction in terms of NaCl molar concentration. [c] Percentage of fraction from total assemblage, expressed in terms of mole % amino acids.

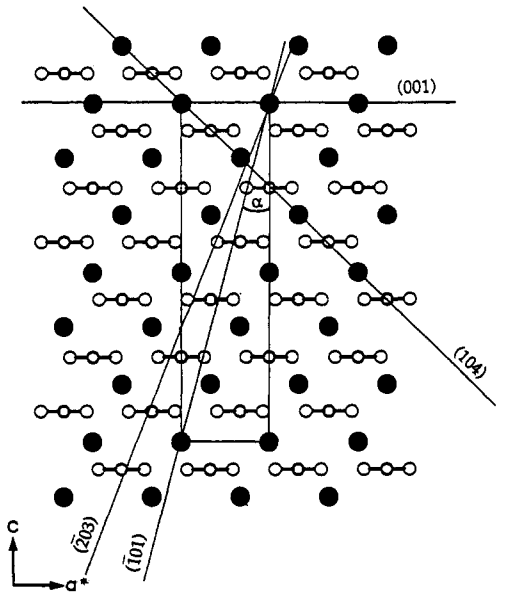

Figure 2. Molecular structure of calcite viewed down the *b* axis. The large atoms are calcium ions. The (104), (001), (1̄01), and (2̄03) faces are viewed edge-on. The (1̄01) and (2̄03) planes are part of the symmetry-related set of planes {011} and {023}, respectively.[17] α is the angle between the *c* axis and the new developed face (see text). In the presence of the sea urchin proteins, the development of faces positioned between the {011} and {023} has been observed.

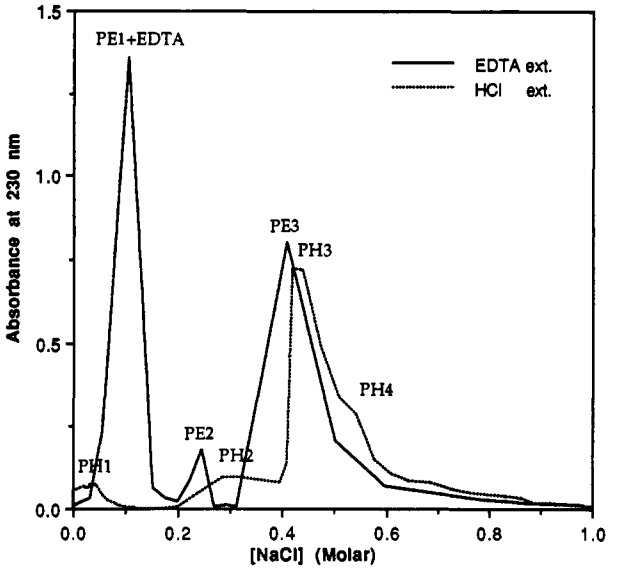

Figure 3. DEAE elution profiles obtained from the mollusk intraprismatic macromolecules extracted with EDTA or HCl.

pigment which is associated with the protein, while the HCl method yields an insoluble protein fraction which tightly binds the pigment.

Table I shows that the mollusk intraprismatic macromolecular assemblage obtained by both extraction techniques has an entirely different amino acid composition than that obtained from the sea urchin spines. When used in the crystallization of calcite, the two assemblages interact in different ways with the growing crystals. The total mollusk intraprismatic extract at concentrations of 1–2 μg/mL produced crystals with curved glassy {104} faces, as well as small and smooth {001} faces. At 3 μg/mL of protein, the crystallization of calcite is inhibited. The few calcite crystals which form are strongly deformed in all directions.[7] The total sea urchin spine extract at concentrations of 2–4 μg/mL induces a mild specific effect, which results in the expression of small new faces roughly parallel to the crystallographic *c* axis.[7,15] Observation of the new faces using SEM revealed microscopic {104} steps, which give the faces a rough appearance. The angles measured between the new {0*kl*} face and the *c* axis of differently affected crystals ranged between 14° and 21°, which corresponds to faces between the {011} set and the {023} set (Figure 2).[17] We will, therefore, consider this new set of faces {01*l*}, with *l* ranging between 1 and 1.5.

Subfractions of the Mollusk Prism Macromolecules. The soluble macromolecules extracted using both EDTA and HCl were further fractionated on an ion-exchange column (DEAE). Figure 3 shows the chromatograms of the eluting fractions. Table II shows the relative concentrations of the different fractions and their amino acid compositions. In both the EDTA and HCl extracts, one fraction contains most of the protein. These major fractions elute at similar NaCl concentrations and have almost identical amino acid compositions, with the sum of Asx and Glx comprising over 80 mole %. Weiner[16] showed that in mollusks most of these residues are acidic. Note also that fraction PH4

(22) Samata, T.; Matsuda, M. *Comp. Biochem. Physiol.* **1986**, *84B*, 200–212.

Table III. Amino Acid Compositions of Fractions from the Total Extracts of Sea Urchin Spines Using EDTA or HCl, after Fractionation on a DEAE Ion-Exchange Column[a]

	EDTA				HCl				
	SE1	SE2	SE3	SE4	SH1	SH2	SH3	SH4	SH5
[NaCl][b]	0.00	0.00–0.15	0.15–0.32	0.32–1.00	0.00–0.16	0.16–0.26	0.26–0.81	0.81–0.95	0.95–1.00
[protein][c]	15	9	61	15	8	23	62	4	3
Asx	10.3	9.2	11.2	13.4	8.7	14.7	15.7	7.2	11.6
Thr	5.5	5.7	6.4	3.9	3.2	5.5	5.6	2.5	5.2
Ser	3.5	4.0	3.4	5.3	3.2	4.0	4.3	3.1	9.6
Glx	12.2	8.5	11.5	11.4	13.6	12.6	11.2	6.4	15.1
Pro	13.7	9.5	8.9	6.2	17.8	12.8	7.5	2.2	2.2
Gly	18.7	28.2	13.9	21.8	28.3	23.1	21.4	64.2	19.5
Ala	10.0	9.4	11.6	12.2	6.6	8.4	13.9	4.0	7.4
Cys	0.1	0.1	0.3	0.1					
Val	5.2	4.2	5.3	3.8	4.8	4.2	3.7	1.5	4.5
Met	2.9	4.2	4.0	4.1	2.8	1.4	4.0	0.7	
Ile	4.7	3.6	5.3	2.8	1.6	2.0	2.9	1.0	3.0
Leu	4.5	3.3	6.6	3.5	1.8	2.2	2.4	1.9	5.5
Tyr	0.4	0.7	1.9	2.0	0.4	1.2	1.7	1.9	4.7
Phe	2.4	2.4	4.1	5.0	2.3	3.1	3.8	1.3	2.6
Lys	2.2	2.7	1.6	1.3	2.0	1.5	0.5	0.6	6.6
His	0.6	0.5	1.8	1.4	0.3	0.7	0.4	0.6	
Arg	3.1	4.0	2.2	1.8	2.6	2.7	1.0	0.9	2.5

[a] Amino acid concentrations are expressed in terms of mole %. [b] Elution position of fraction in terms of NaCl molar concentration. [c] Percentage of fraction from total assemblage, expressed in terms of mole % amino acids.

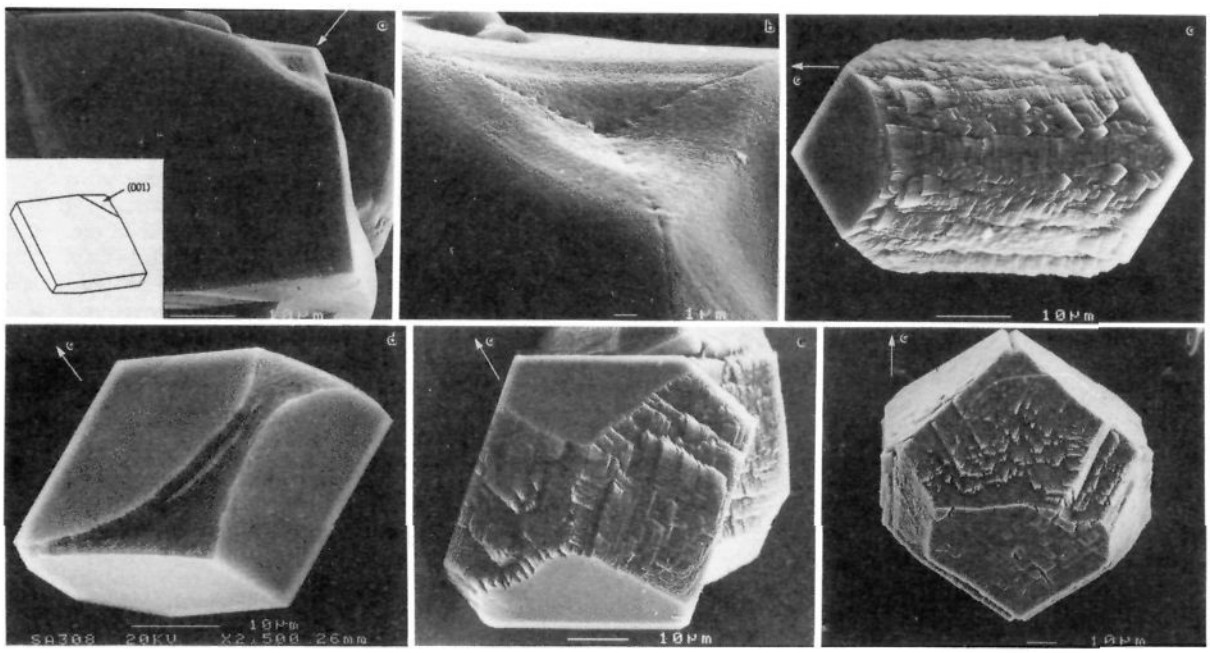

Figure 4. (a) Synthetic calcite crystal grown in the presence of fraction PE3 (2 μg/mL) of the EDTA-extracted macromolecules from the mollusk prisms. Similar crystals were obtained in the presence of PH3 and PH4. The small (001) face is indicated by the arrow. Insert: computer simulation of the morphology of a crystal expressing the (001) face. (b) Enlargement of the (001) face, showing its smooth character. (c) Synthetic calcite crystal grown in the presence of traces of EDTA expressing the nonspecific inhibition of growth in the envelope of planes (*hk*0). (d) Synthetic calcite crystal grown in the presence of fraction PH1 of the HCl-extracted intraprismatic proteins (2 μg/mL). The new {01*l*} faces are the same as those expressed by calcite crystals grown in the presence of the total sea urchin extracted proteins.[15] (e) Synthetic calcite crystal grown in the presence of fraction SH2 (2 μg/mL) of the HCl-extracted sea urchin spine proteins. Similar crystals were obtained from fractions SH1 and SH4 and subfractions from the HPLC separation of SE3. Note the well-developed {01*l*} faces (in this crystal α is close to 21°, which corresponds to *l* with a value of 1.5) and the texture of the face expressing both {104} and {01*l*} steps. (f) Synthetic calcite crystals grown from a 30 mM [Ca^{2+}] and 6 mM [Mg^{2+}] solution. Note the well-developed {011} faces with {011} steps.

in the HCl extract is much richer in Glx than Asx. The fractions of both extraction methods eluting at low ionic strength (PE1, PE2, PH1, and PH2) are much less acidic than the fractions eluting at higher ionic strength, and their compositions resemble those of the sea urchin spine extracted proteins (Table III).

When calcite crystals were grown from solutions containing as little as 2 μg/mL of fractions PE3, PH3, or PH4, the resultant crystals expressed smooth {001} faces (Figure 4a,b). Fraction PE1, which elutes from the column in the vicinity of the remaining EDTA, did not express {001} faces. Its presence in solution induced the expression of an envelope of {*hk*0} faces. EDTA alone in solution interacts nonspecifically with growing calcite crystals parallel to the *c* axis, expressing the same envelope of faces (Figure 4c). The effect of PE1 is, therefore, probably due to the presence of traces of EDTA. This may mask any specific effect, if present, due to the proteins in directions that are part of the same envelope. The corresponding fraction PH1 from the HCl extract, which does not contain traces of EDTA, induces the formation of {01*l*} faces analogous to those induced by the total sea urchin extract (Figure 4d).

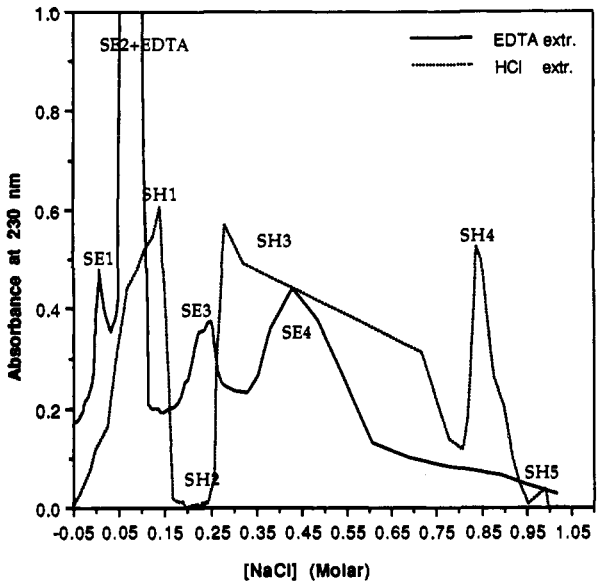

Figure 5. DEAE elution profiles obtained from the sea urchin spine macromolecules extracted with EDTA or HCl.

Subfractions of the Sea Urchin Spine Macromolecules. Figure 5 shows the separation of the EDTA and HCl extracts of sea urchin intracrystalline proteins on a DEAE ion-exchange column. Table III shows the distribution of protein in each fraction as determined by amino acid analyses and their amino acid compositions. The two chromatograms in Figure 5 are rather similar except that the HCl-extracted components are more tightly bound to the column. This phenomenon has also been observed by Samata.[23] In both cases, a fairly large amount of protein elutes in the void volume, and both separations include one fraction which contains about 65 mole % of all the protein eluting from the column. Surprisingly, the amino acid compositions of the various fractions do not reveal exceptional differences apart from fraction SH4, which contains an unusually high amount of Gly.

The macromolecules were assayed in terms of their interactions from solution with growing calcite crystals. When 2 μg/mL of fractions SE1, SE3, and SE4 was introduced into the crystallizing solution, the calcite crystals were unaffected. Only when 5 μg/mL of SE3 or SE4 was used, were small {0*kl*} faces observed similar to those expressed by the total sea urchin extract. Fraction SE2 caused the typical formation of an envelope of {*hk*0} faces due to the presence of traces of EDTA.

The effect of the fractionated HCl-extracted proteins on the growth of calcite is considerably stronger than that of the corresponding EDTA fractions. Two micrograms per milliliter of fraction SH3 was sufficient to produce small {01*l*} faces. Fractions SH1, SH2, and SH4 cause the expression of well-developed {01*l*} faces at this concentration (Figure 4e). It is noteworthy that the largest fractions of both extracts, namely SE3 and SH3, are relatively inactive, yielding crystals which are only mildly affected even at high concentrations of protein.

The major component of the EDTA extract, SE3, was further fractionated by HPLC ion-exchange chromatography (Mono Q), yielding three fractions. The three subfractions thus obtained also induce the formation of {01*l*} faces in newly crystallized calcite. However, a concentration of 2 μg/mL is sufficient to produce crystals with large, well-developed {01*l*} faces similar to those in Figure 4e.

Interaction of Magnesium Ions with Growing Calcite Crystals. Magnesium ions were introduced into solutions of growing calcite crystals in various concentrations and with varying magnesium/calcium proportions. We observed that magnesium specifically interacts with {011} faces of calcite, as shown by the fact that the angle between the new face and the *c* axis is 13°–14° (Figure 4f). The angle is independent of magnesium concentration or the size of the face. These new faces are composed of many {011} steps. The ratio magnesium/calcium and the absolute concentration of the magnesium ions affect the density of these steps. As the magnesium ion concentration is increased, the {011} face becomes both more pronounced and more densely stepped. The optimum proportion of magnesium/calcium to produce crystals of good size with well-developed {011} faces was found to be 1/5. Below a concentration of 1.5 mM magnesium, the new faces are not observed. At calcium ion concentrations above 50 mM, the calcite crystals are unaffected, even when the magnesium/calcium ratio is maintained at 1/5.

Discussion

In this study we show how the partially purified components from two biogenic skeletal elements interact differently *in vitro* with growing calcite crystals. The mollusk shell prisms contain macromolecules which can interact specifically with two different sets of crystal faces, {001} and {01*l*}, whereas the sea urchin proteins only interact with the sets of faces roughly parallel to the *c* axis, {01*l*} ($1 < l < 1.5$). Magnesium, a major ionic component of the sea urchin skeleton, interacts specifically with the {011} set of crystal faces, which is closely related to the {01*l*} faces.

Sea Urchin Protein/Calcite Interactions. Earlier studies showed that the total ensemble of sea urchin macromolecules preferentially interacted with crystal faces roughly parallel to the *c* axis.[15] The newly expressed faces were rough and not well defined, as a result of locally developed {104} steps. Careful crystallization using partially purified fractions resulted in crystals with better developed faces, although the presence of {104} steps persisted. A detailed analysis of the identity of the new faces showed that they define an angle of between 14° and 21° with the *c* axis, depending upon the concentration of protein used in the experiment and its degree of purity. Surprisingly, the same is true for all the different fractions separated by ion-exchange chromatography. Clearly, a precise interpretation of the interactions at the molecular level, leading to the development of the {01*l*} faces, must be postponed until more information is available on the sequence and conformation of the interacting domains of the protein. We note that this set of faces is close to parallel to the *c* axis. Consequently, the carbonate ions emerge almost perpendicular to the face, with two oxygens available for binding (Figure 2). This orientation was found to be particularly favorable for interaction with acidic matrix macromolecules.[14,24] The roughness of the face may be due to intrinsic instability of the crystal plane, associated with the formation of the more stable {104} steps. A flexible macromolecule, adsorbed through a specific domain on one crystal plane, may also influence adjacent directions, leading to the expression of faces that are a combination of less stable and more stable planes.

The physical basis for the separation of the macromolecules is not clear, as all the fractions have a similar amino acid composition. It may be based on different conformational states, aggregation, or a different degree of side-chain substitution. Note too that calcite crystals overgrown on various echinoid skeletal elements also express the same {01*l*} faces.[18] Under these conditions the proteins released from the biogenic elements undergo minimal structural modification prior to being readsorbed on the newly crystallizing calcite. Thus proteins after separation interact with calcite crystals in a manner similar to those in close-to-native conditions, strengthening the notion that we are monitoring parameters relevant to the system *in vivo*. Measurements of crystal texture showed a reduction in coherence length perpendicular to the *c* axis; a result consistent

(23) Samata, T. *The Veliger* **1990**, *33* (2), 190–201.

(24) Addadi, L.; Moradian, J.; Shay, E.; Maroudas, N. G.; Weiner, S. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 2732–2736.

with the intercalation of proteins parallel to the *c* axis.[11] We are aware, on the other hand, that the environmental growth conditions are drastically different in our *in vitro* experiments from those *in vivo*, possibly leading to different expressions of the same parameters and interactions.

Mollusk Prismatic Layer Proteins/Calcite Interactions. In contrast to the case of the sea urchin, a study of the intraprismatic mollusk shell macromolecules revealed two families of macromolecules, each having a characteristic amino acid composition and displaying distinct specific interactions with growing calcite crystals. The minor fraction has an amino acid composition similar to that of the sea urchin spine. It induces the formation of the same {01*l*} faces as the sea urchin macromolecules. The major fraction of this ensemble is rich in carboxylate groups, due to its unusually high Asp and Glu content. This fraction induces the formation of smooth {001} faces. Calcite crystals overgrown on isolated prisms express both the {01*l*} and {001} faces, apparently corresponding to the independent interactions of both families of macromolecules with the growing calcite crystals.[18] When the total protein extract is present in a solution of newly crystallizing calcite, the effect of the two protein fractions combines to result in almost total nonspecific inhibition. This prevents detection of the separate effects, that could be achieved only after isolation of the fractions.

At the molecular level, the {001} planes of calcite are characterized by alternating layers of calcium and carbonate ions, producing homoionic {001} faces (Figure 2). These faces, being either totally cationic or anionic over large domains, are intrinsically unstable. They may be stabilized by electrostatic interactions between the calcium ions on the crystal surface and the carboxylates on the proteins. This kind of interaction was shown to induce oriented crystal nucleation from the {001} plane, when analgous acidic proteins (extracted from *Mytilus californianus* shells) were preadsorbed on a rigid substrate.[14,25] Lightly etched individual shell prisms reveal concentrations of organic material on planes parallel to {001}, suggesting the presence of macromolecules adsorbed on these planes *in vivo*.[26] Crystal texture measurements of the same biogenic prisms showed anisotropic reduction of coherence length in the [001] direction, also in agreement with the presence of macromolecules intercalated on the {001} planes.[11]

Magnesium Effect. Direct recrystallization of calcite from a solution of dissolved sea urchin spines resulted in crystals expressing well-developed {011} faces. We attribute their formation to the presence of magnesium ions. We found in fact that 5 mole % magnesium is sufficient to cause the development of small {011} faces even in the absence of proteins. As the magnesium ion concentration increases, pronounced inhibition causes the formation of large, stepped {011} faces. An examination of the surface texture of the newly expressed face at high magnification using a scanning electron microscope shows the presence of only {011} steps. When the magnesium/calcium ratio exceeds 4/1, aragonite is formed.[27,28] The magnesium effect depends on the absolute concentration of calcium as well. When the calcium concentration exceeds 50 mM, the magnesium effect is not observed, even though the magnesium/calcium ratio is maintained constant. Under these conditions, the calcite surface is presumably saturated with calcium ions, reducing their ability to adsorb magnesium.

The mechanism by which magnesium ions interact with growing calcite is not clear. Energy calculations showed a marked preference for adsorption of magnesium on the {100} planes.[29] Experimentally we have, however, never observed the formation of these faces. We suggest that this may be due to the presence of a tightly bound hydration layer around each magnesium ion in solution, a property that distinguishes it from calcium ions. The extent of inhibition of growth at the site of adsorption would thus be determined by the dehydration of the magnesium ions. The {011} faces may be good candidates for adsorption, because they are composed of alternating anionic and cationic patches. The cations are far enough from each other to accommodate the incoming large hydrated ions (Figure 2).

It is not known how protein and magnesium may cooperate in the formation of the skeletal elements *in vivo*. We point out that biogenic calcite may occlude up to 45 mole % magnesium.[30] The maximum achievable incorporation *in vitro* under close to physiological conditions is 6 mole %. One possibility is that in the biological system magnesium is delivered to the crystal surface already partially dehydrated, thus lowering the activation energy for adsorption of the ion to the surface and the consequent inhibition of crystal growth. This might be conceivably achieved through precomplexation of magnesium with chelating molecules or macromolecules. We note also that the {011} and {01*l*} faces developed in the presence of magnesium and sea urchin protein, respectively, are spatially closely related. Magnesium could thus contribute to the development of active sites for protein adsorption on planes that are normally not expressed during crystal growth.

In summary, the isolation of intracrystalline components has allowed the evaluation of their separate effects on calcite crystal growth. Each separate mode of interaction may infer a specific role in the regulation of biological crystal growth. Organisms are clearly able to manipulate these components in a much more sophisticated manner by secreting and introducing them at will in different locations in a perfectly orchestrated sequence in time and space. We cannot reproduce the microenvironment of crystal growth *in vivo*. We can try to reach a better understanding of each set of interactions at the molecular level. The comparison between the effect of proteins from the same or from different organisms and their correlation with the known characteristics of the biogenic minerals will hopefully throw some light on the structure–function relations involved in the formation of these peculiar composite materials.

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(25) Addadi, L.; Weiner, S. *Mol. Cryst. Liq. Cryst.* **1986**, *134*, 305–322.
(26) Cuif, J. P.; Gautret, P.; Marin, F. In *Mechanisms and Phylogeny of Mineralization in Biological Systems*; Suga, S., Nakahara, H., Eds.; Springer-Verlag: Tokyo, 1991; pp 391–395.
(27) Lippmann, F. In *Sedimentary Carbonate Minerals*; von Engelhardt, W., Hahn, T., Roy, R., Wyllie, P. J., Eds.; Springer-Verlag: Berlin, 1973; pp 107–116.
(28) Kitano, Y. *Bull. Chem. Soc. Jpn.* **1962**, *35*, 1973–1980.
(29) Titiloye, J. O.; Parker, S. C.; Osguthorpe, D. J.; Mann, S. *J. Chem. Soc., Chem. Commun.* **1991**, 1494–1496.
(30) Schroeder, J. H.; Dwornik, E. J.; Papike, J. J. *Bull. Geol. Soc. Am.* **1969**, *80*, 1613–1616.