Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 2732–2736, May 1987
Biophysics

A chemical model for the cooperation of sulfates and carboxylates in calcite crystal nucleation: Relevance to biomineralization

L. ADDADI*, J. MORADIAN*, E. SHAY†, N. G. MAROUDAS‡, AND S. WEINER†

Departments of *Structural Chemistry and †Isotopes, The Weizmann Institute of Science, Rehovot 76100, Israel; and ‡Faculty of Medicine, Technion, Haifa, Israel

Communicated by H. A. Lowenstam, December 15, 1986

ABSTRACT **Acidic matrix macromolecules involved in regulation of biological crystal growth often contain aspartic acid-rich domains and covalently bound sulfated polysaccharides. We propose that sulfates and β-sheet structured carboxylates cooperate in oriented calcite crystal nucleation. The sulfates concentrate calcium, creating the supersaturation necessary for nucleation on the structured carboxylate domains. An artificial model, composed of sulfonated polystyrene surfaces and adsorbed β-sheet poly(aspartate), demonstrates that the two components indeed act cooperatively with respect to two independent assays, both by induction of calcite nucleation off the (001) plane and by calcium association. Evidence is presented that a purified organic matrix acidic glycoprotein from mollusk shells may behave *in vitro* in a similar way.**

Crystal formation by organisms is commonly controlled by an array of extracellular proteins and polysaccharides. In skeletal mineralization these macromolecules generally form a mold or framework in which the crystals grow. The macromolecules on the surfaces of this "organic matrix" are most directly involved in regulating crystal nucleation and growth. They are characteristically acidic in nature (1). The polysaccharides are generally sulfated and carboxylated (2).

Addadi and Weiner (3, 4) demonstrated that acidic glycoproteins from mollusk shells, in the β-sheet conformation, are able to interact from solution specifically with certain crystal faces of calcium dicarboxylates that have a common stereochemical property. This was explained in terms of the appropriate orientation and disposition of the aspartate side-chain carboxylates emerging from the planar protein β-sheets, which create extended structured calcium-interacting domains. When adsorbed onto a solid substrate, these mollusk proteins can induce oriented calcite crystal formation from the (001) face, which is stereochemically equivalent to the interacting faces of the model calcium dicarboxylates. This orientation of calcite—namely, with the *c* axis perpendicular to the surface of nucleation—is also the one most frequently found in biological mineralization. We subsequently noted that adsorbed poly(aspartic acid) alone, although in the β-sheet conformation, is not able to induce (001)-oriented calcite nucleation, and we therefore concluded that some additional factor is required.

In mollusks, there is good evidence showing that sulfur, presumably in the form of sulfate, is present at the nucleation site (5, 6). Sulfated polysaccharides from cartilage have a strong calcium-concentrating effect but act as crystal nucleation and growth inhibitors rather than nucleators (7). Under these conditions the calcium ions show a nearly ideal Donnan distribution (8), indicating that they are not bound on a one-to-one basis to sulfate groups. Such an "ionotropic effect" (9) was in fact proposed as a mechanism for inducing crystal nucleation in mollusk shell formation (6). We therefore hypothesized that in our *in vitro* experiments (3, 4) the sulfate groups of the polysaccharides, which are covalently bound to the protein backbone, concentrate calcium. This creates the local supersaturation necessary for nucleation of calcite on the structured carboxylate domains.

To test this hypothesis we created an artificial model substrate for crystal growth composed of sulfonated polystyrene films (10) to which poly(aspartate), in the β-sheet conformation, is adsorbed. The separate and combined effects of the two elements of the system are tested. Two independent "assays" are used: induction of (001)-oriented calcite nucleation and calcium association. We show that indeed sulfonates and structured carboxylates act cooperatively with respect to both assays, and we present evidence suggesting that a purified organic matrix acidic glycoprotein from mollusk shells behaves *in vitro* in a similar way.

MATERIALS AND METHODS

Materials. Untreated polystyrene films (100-μm thick) were obtained from Sterilin (Teddington, Middlesex, England). Poly(aspartic acid) (n = 52, average M_r 6000) was from Bioyeda (Rehovot, Israel) and poly(acrylic acid) (n = 70, average M_r 5000) was from Aldrich. Poly(glutamic acid) (n = 140, average M_r 20,000) was synthesized as described (11). Spectrapor dialysis membranes with M_r cut-off 6000–8000 were from Spectrum Medical Industries (Los Angeles). Falcon cell culture dishes (3 cm in diameter) were obtained from Becton Dickinson. The water used throughout was double-distilled.

Polystyrene Films. Polystyrene films were sulfonated to varying extents by treatment with 98% H_2SO_4 at room temperature for different periods of time. Film strips of 10 × 30 cm were wrapped around the internal surface of a beaker containing the acid with constant stirring. The films were thoroughly washed with water, dialyzed for at least 10 hr against 1 mM $CaCl_2$ to remove residual acid, redialyzed against water, and then dried. Some of the film was incubated for 24 hr in a 1 mM $CaCl_2$, 0.2 μM polymer, or 0.5 μM protein solution at pH 7.0, then washed with water, and dried. The film surfaces were examined by attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy using a KRS-5 prism at an angle of 45° in a Nicolet MX-1 spectrometer. Data were collected for 2 hr. The spectra of surface-bound sulfonate groups (1008, 1035, 1129, and 1176 cm^{-1}) (12) and adsorbed polymer (1500–1800 cm^{-1}) were obtained by subtracting out the appropriate reference polystyrene film spectrum. Films were examined in a Phillips 505 scanning electron microscope after coating with gold or carbon.

Crystallization of Calcite on Films. Calcite crystals were grown by slow diffusion (about 4 days) of $(NH_4)_2CO_3$ vapor into cell culture dishes containing 2 ml of 7.5 mM $CaCl_2$ (pH 7) in a desiccator (20–40 cm in diameter). $(NH_4)_2CO_3$ powder

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: ATR-FTIR, attenuated total reflection-Fourier transform infrared.

was placed in four vials. Slow diffusion was achieved through five or six needle holes in the lids of the vials and in the Parafilm covering the culture dishes. The films (2 × 2 cm) were stuck to the bottom of the culture dishes with cyanoacrylate glue. After crystallization the films were air dried and crystals (≈40–200 μm) were counted (double blind) under a microscope (×50). The (001)-oriented crystals were identified by the perfect hexagonal symmetry in two-dimensional projection down the *c* axis. Careful examination of the unoriented crystals shows that they do not have developed (001) faces and are therefore not crystals that were oriented and then mechanically dislodged. All results are averaged over >10 triplicate experiments.

Calcium Association to the Films. Films sulfonated for 0.5 min, 1 hr, and 24 hr with and without adsorbed poly(aspartic acid) were incubated for 24 hr at room temperature in 2-ml solutions of $CaCl_2$ (pH 7.0) ranging in concentration from 10^{-7} M to 10^{-4} M and containing about 4×10^5 cpm of ^{45}Ca. Concentrations of ^{45}Ca after equilibration were measured in a Beckman LS 7500 liquid scintillation counter. Each experiment was performed on two parallel samples and each sample was measured in duplicate. Calcium association was calculated from the differences in radioactivity between solutions equilibrated with sample films [sulfonated ± adsorbed poly(aspartate)] and reference (0.5-min sulfonated) films. The standard error was calculated from three to six independent experiments.

Acidic Organic Matrix Proteins. These were extracted from the calcitic prismatic layer of the bivalve *Mytilus californianus* and separated from other matrix proteins using a reversed-phase C_{18} cartridge. The "A" fraction (13) was used. One randomly chosen macromolecule, present in relatively large amounts, was purified to homogeneity by high-performance liquid chromatography (HPLC) as described (13). The macromolecule elutes from the column with 30% acetonitrile. Carboxylate groups of the acidic glycoproteins were modified by the Hoare–Koshland method (14). Sulfate contents were measured by turbidimetry after solvolitic desulfation (15) and were measured independently by ion chromatography (16) using a Wescan model 215 ion chromatograph. For the latter the samples were oxidized overnight at 70°C in 0.5 ml of H_2O_2 and the residue was redissolved in water. Amino acid composition analyses and FTIR spectroscopy of protein samples were performed as described (13). The apparent molecular weight of the purified protein was determined by gel electrophoresis in 14% acrylamide as described by Laemmli (17); protein was stained with Stains-All followed by silver stain (18).

RESULTS

Nucleation of Calcite on Sulfonated Polystyrene Films. Fig. 1 shows the results of two independent calcite crystallization experiments on polystyrene films sulfonated to different extents. The number of calcite crystals progressively increases on films treated with H_2SO_4 up to 8 hr and then decreases, despite the fact that the sulfonate contents of the films steadily increase (8-hr films have half the amount of sulfonate as 24-hr films), as demonstrated by FTIR and x-ray dispersive analysis. The percentage of crystals nucleated off the (001) plane is also maximal on 8-hr films and then decreases. Films treated for 1 hr and 24 hr by H_2SO_4 have ≈7% oriented calcite crystals, whereas the 8-hr treated films have 60–65%. The remaining crystals usually lie on their (104) cleavage rhombohedral faces, which are the only faces developed in the absence of any external agent. The dramatic increase in the proportion of oriented calcite crystals on 8-hr films may be due either to specific chelation of calcium between groups of closely spaced sulfonate moieties on a rigid substrate or to a nonspecific electrostatic effect. The latter could conceivably induce oriented nucleation, as calcite is composed of alternating layers of calcium and carbonate in the plane perpendicular to the *c* axis. We exclude the first option, as calcite crystallization on 8-hr films in the presence of a 10-fold excess of sodium over calcium results in a drastic decrease in the number of crystals formed and the proportion of oriented crystals.

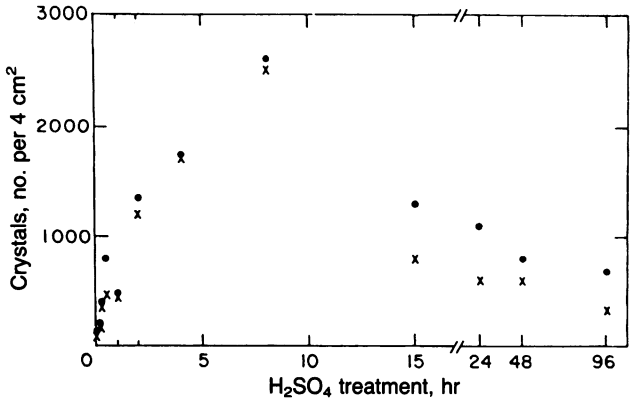

FIG. 1. Crystallization of calcite on sulfonated polystyrene films: dependence of the number of crystals on the length of H_2SO_4 treatment. ● and ×, two independent sets of crystallization performed in parallel.

Note that experiments of this type are very sensitive to the presence of water during the sulfonation step, which oxidizes the film surface. The oxidation products, when present, can be detected by ATR-FTIR spectroscopy and substantially alter the crystal nucleation properties of the films. The absolute number of crystals formed on the films is also strongly dependent on the rate of $(NH_4)_2CO_3$ diffusion and on the nature (crystallinity, etc.) of the starting films (19) (a more detailed characterization of the films will be published separately). For this reason, sets of increasingly sulfonated films were all exposed to identical conditions. Whereas the absolute number of crystals may vary from experiment to experiment, the trends are always the same.

Nucleation of Calcite on Polystyrene Films with Adsorbed Poly(aspartate). Poly(aspartic acid) in a 5 mM $CaCl_2$ solution at pH 7.0 partially assumes the β-sheet conformation in solution (approximately 60:40 random coil: β-sheet conformation), as demonstrated by circular dichroism (20). The poly(aspartate) solution was adsorbed onto polystyrene films that had been sulfonated for 0.5 min to give them some hydrophilic character. Infrared spectra of these air-dried films show that poly(aspartate) does adsorb (Fig. 2*A*). The amide I absorption peaks at ≈1640 cm^{-1}, showing that some of the adsorbed poly(aspartate) is in the β-sheet conformation (21). Very few calcite crystals nucleate on these films, with or without adsorbed poly(aspartate). Furthermore, only about 3% of the few crystals formed are oriented with their (001) faces in the plane of the film (Table 1). In competitive experiments between 24-hr sulfonated films and 0.5-min sulfonated films with adsorbed poly(aspartate), almost all of the crystals formed on the 24-hr sulfonated films, showing that adsorbed poly(aspartate) alone is a very bad nucleator of calcite.

Demonstration of Cooperativity by Nucleation. Films sulfonated for 1 hr and 24 hr nucleate relatively few crystals (Fig. 1) as compared to 8-hr films, and only about 7% of the formed crystals are (001) oriented. We therefore adsorbed poly(aspartic acid) in the presence of calcium onto the 1-hr and 24-hr films. The polypeptide is still partially in the β-sheet conformation (Fig. 2*B*) and approximately the same amount of material is adsorbed on these films as in 0.5-min treated films (the control) (Fig. 2*B*). Calcite crystals were then grown on the films. The 1-hr treated films did not show any increase

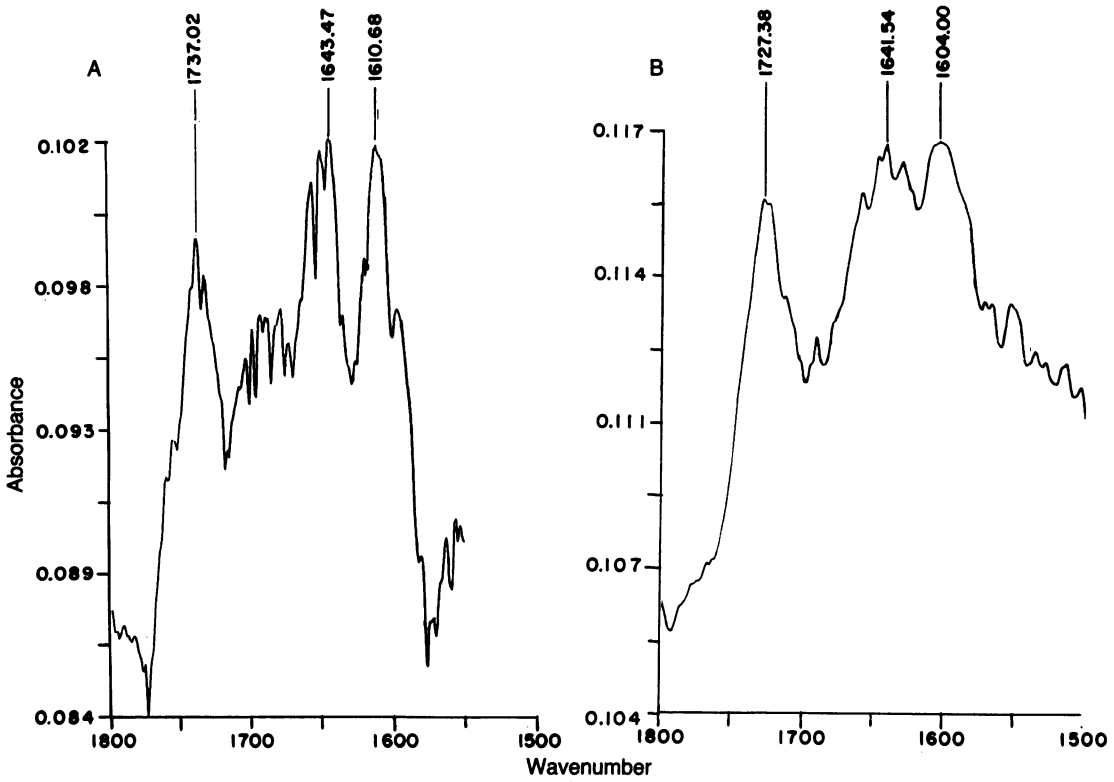

FIG. 2. ATR-FTIR spectra of poly(aspartic acid) adsorbed on 0.5-min sulfonated films (*A*) and 24-hr sulfonated films (*B*).

in the percentage of oriented crystals, whereas the 24-hr films had approximately three times as many (001)-oriented crystals (25%) as the 24-hr film without poly(aspartic acid) (7%) (Table 1 and Fig. 3). We conclude that in the case of the 24-hr films, but not of the 1-hr films, the sulfonate groups and the poly(aspartate) cooperate to induce (001)-oriented calcite nucleation. Twenty-four-hour films incubated with five times as much poly(aspartic acid) (1 μM rather than 0.2 μM) induced spherulites or poorly oriented crystal assemblies to form. We infer from both observations that an optimal proportion of sulfonates and carboxylates is necessary for (001)-oriented calcite nucleation. The β-sheet conformation of the poly(aspartic acid) is also essential, as carboxylate-rich poly(glutamic acid) and poly(acrylate), which do not assume the ordered conformation but do adsorb onto the films, do not increase the percentage of (001)-oriented crystals.

Demonstration of Cooperativity by Calcium Association on Polystyrene Films. Films sulfonated for 24 hr and to which poly(aspartate) has been adsorbed associate more than twice as much calcium as 24-hr sulfonated films without poly(aspartate) (Fig. 4*A*). Reference films with adsorbed poly(aspartate) but without sulfonate groups do not reveal any substantial affinity for calcium up to concentrations of $\approx$0.1 mM. Films sulfonated for 1 hr showed much lower calcium association values than the highly sulfonated ones and, significantly, no apparent change was introduced by the presence of adsorbed poly(aspartate), an observation consistent with the nucleation experiments. These calcium association experiments thus independently demonstrate that cooperativity does take place between the sulfonate and carboxylate ligands, given a high enough concentration of the former.

Organic Matrix Glycoproteins and Oriented Calcite Nucleation. An assemblage of aspartic acid-rich proteins from mollusk shells adsorbed on solid substrates is able to induce (001)-oriented calcite nucleation (3, 4). To assess the possible relevance of the cooperative effect in this process, we first determined the sulfate content of the assemblage. Measurement by turbidimetry and anion chromatography showed 69 $\pm$ 7 and 74 $\pm$ 7 residues of sulfate per 100 amino acids, respectively. The carboxylate groups of the assemblage of mollusk glycoproteins in solution and adsorbed onto the films were then blocked by reaction with glycine methyl ester (14). In both cases oriented calcite nucleation was suppressed (Table 1). One randomly chosen, but quantitatively abundant acidic glycoprotein was purified by HPLC from the assemblage, which contains about 20–30 different macromolecules. This glycoprotein contains 72 $\pm$ 12 sulfates per 100 amino

Table 1. Percentage of (001)-oriented calcite crystals nucleated on polystyrene films with different treatments

Polystyrene film	Adsorbed polymer	(001)-oriented calcite crystals, %	
		Without polymer	With polymer
Sulfonated 0.5 min	Poly(aspartic acid)	3.0 $\pm$ 0.5	2.5 $\pm$ 1.0
Sulfonated 1 hr	Poly(aspartic acid)	7.5 $\pm$ 3.0	8.5 $\pm$ 4.0
Sulfonated 24 hr	Poly(aspartic acid)	7.0 $\pm$ 4.0	25 $\pm$ 7.0
Culture dish	Pure protein	7.4 $\pm$ 1.5	14.6 $\pm$ 3.9
Sulfonated 0.5 min	Protein assemblage	3.0 $\pm$ 0.5	17.5 $\pm$ 6.5
Sulfonated 0.5 min	Protein assemblage (blocked carboxylate)	3.0 $\pm$ 0.5	5.7 $\pm$ 0.6

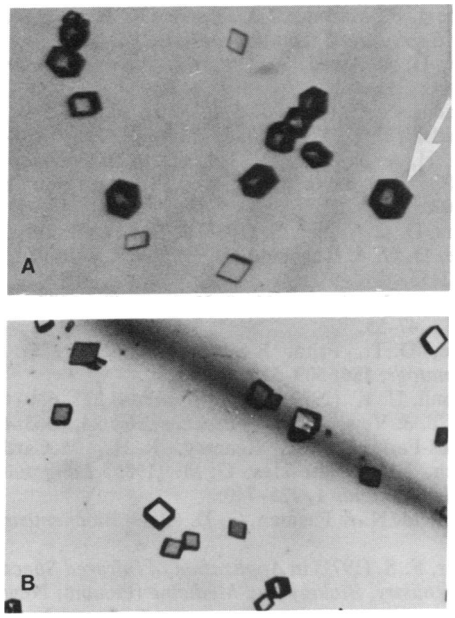

FIG. 3. Optical microscope pictures of calcite crystals grown on 24-hr sulfonated films. (*A*) With adsorbed poly(aspartic acid). (*B*) Without adsorbed poly(aspartic acid). The arrow indicates a typical crystal nucleated from the (001) plane.

acids. The most abundant amino acids in the protein moiety are aspartic acid/asparagine (30.1%), threonine (6.0%), serine (9.6%), glutamic acid/glutamine (25.0%), proline (9.1%), glycine (5.3%), and lysine (4.0%). The apparent M_r of the glycoprotein estimated by gel electrophoresis is about 15,000. The FTIR spectrum of the macromolecule shows that it contains polysaccharide. Crystal nucleation experiments with this protein adsorbed onto cell culture dishes show that it induced approximately twice as many (001)-oriented crystals as were formed on the control substrate (14% as opposed to 7%). We show, therefore, that this single macromolecular complex that contains sulfate and carboxylate ligands is itself able to induce oriented calcite crystal growth and that this does not occur if the carboxylate groups are blocked.

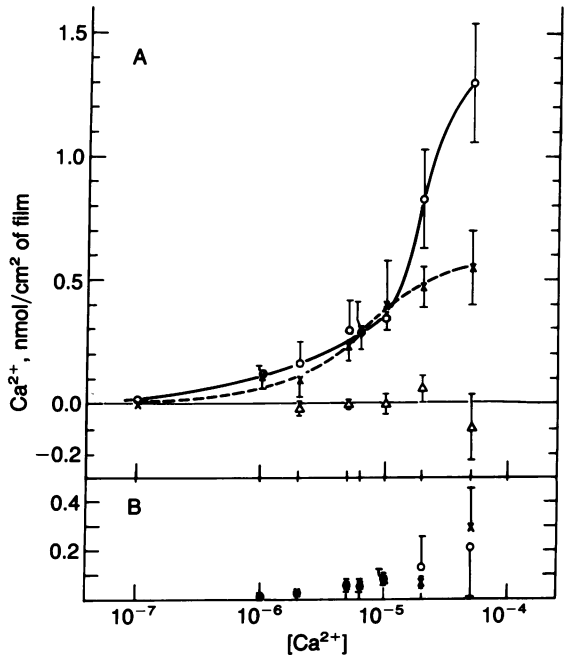

FIG. 4. Calcium association on films expressed in nmol of Ca per cm^2, as a function of calcium concentration in solution. (*A*) ×, Twenty-four-hour sulfonated films; ○, 24-hr sulfonated films with adsorbed poly(aspartic acid); △, 0.5-min sulfonated films with adsorbed poly(aspartic acid). (*B*) ×, One-hour sulfonated films; ○, 1-hr sulfonated films with adsorbed poly(aspartic acid).

DISCUSSION

We have demonstrated that in a model system sulfonate and carboxylate ligands do cooperate to induce oriented calcite nucleation. This occurs on 24-hr sulfonated films to which poly(aspartate) in the β-sheet conformation and in the appropriate amount is adsorbed.

Morphological examination of the film surfaces in the scanning electron microscope shows that the 24-hr treated films have a highly irregular etched surface, whereas the 1-hr and 8-hr film surfaces are smooth. Furthermore, the measured amount of calcium associated with the 24-hr film (0.55 nmol/cm^2) corresponds to an amount of sulfonates that exceeds that required for total surface coverage. Following the observation that flexible polyelectrolytes act as crystal nucleation inhibitors (22) and having ourselves experimentally proved that polystyrene sulfonate in solution is a very powerful inhibitor of calcite nucleation and growth, we suggest that the decrease in calcite-nucleating ability (oriented and unoriented) of 24-hr films is due to loss of rigidity of the film surface when subjected to prolonged sulfonation. Analogous effects have been shown for cell adhesion to polystyrene surfaces (10).

A literature survey of the distribution of ion-binding ligands in mineralized tissues in which crystal growth is controlled shows that all of the tissues, for which data are available, contain carboxylate-rich acidic proteins (1). In the few cases in which their structures are known, they adopt the β-sheet conformation (12, 23). Sulfur, shown in some cases to be in the form of sulfate, is present in calcitic coccolithophoridae (24), foraminifera (25), calcitic and aragonitic shells of mollusks (6, 26), phosphatic brachiopods (27), and vertebrate bone (28). The sulfate is associated with polysaccharides, which *in vivo* usually do not adopt a rigid ordered structure (29). In all of these tissues, the crystals are oriented in preferred directions. It seems, therefore, that the highly sulfonated substrate (24-hr films) to which poly(aspartate) in the β-sheet conformation is adsorbed may be the best analogy to these biological systems, raising the possibility that cooperativity is required *in vivo* for oriented crystal nucleation. The purified mollusk shell glycoprotein *in vitro* appears to orient calcite crystals by a similar mechanism. In this case the two cooperative ligands are part of the same macromolecular complex. This is probably not a prerequisite and it is quite possible that cooperativity in crystal nucleation can occur between, say, sulfate groups of proteoglycans and carboxylates of acidic proteins. It is also conceivable that ligands such as phosphate could function in association with carboxylates to orient crystals during nucleation, as has been proposed by Lee and Veis (30). The electrostatic effect that is responsible for (001)-oriented calcite nucleation on the 8-hr films might also have its counterpart in biology.

We attribute the basis for the cooperative mechanism to the very different ways in which the carboxylates and the sulfates (or sulfonates) interact with calcium ions. The carboxylates, although relatively weak binders of calcium, are part of an ordered protein structure and are thus able to bind calcium ions in an ordered array [see discussions in Addadi and Weiner (3, 4)]. The sulfates strongly associate with calcium. However, being part of an oligosaccharide structure, they act as a polyion, generating a strong field to which the calcium ions are drawn, without being bound to specific groups. In this scheme, the ionotropic effect (6) represents just the first stage of crystal nucleation. High- and

low-affinity calcium binding sites have been measured on mollusk organic matrix components in solution (31). It is however difficult to directly relate these observations to the results reported here, because of the different chemical (sulfonates instead of sulfates) and physical (macromolecule in solution instead of adsorbed on a rigid surface) states of the species measured.

We emphasize that the *in vitro* experiments are very different from the *in vivo* environments. Hence, the significance of using sulfonate rather than sulfates, the effect of an excess of sodium over calcium on nucleation, and the contribution of the protein structures and organization to crystallization are just some of the issues that still need to be carefully evaluated. However, the fact that so many mineralized tissues contain sulfate and carboxylate ligands as well as the *in vitro* demonstration that cooperativity is required for one matrix macromolecule to orient calcite crystals suggest that this phenomenon may be widespread in biology.

We thank Y. Jakobson for synthesis of poly(glutamic acid), Y. Adar for performing the first experiments, and M. Lahav, L. Leiserowitz, W. Traub, and Z. Berkovitch-Yellin for valuable discussions. This work was supported by Minerva Foundations grants to L.A. and S.W., by the Fund for Basic Research administered by the Israel Academy of Sciences, and, in part, by funds granted to L.A. by the Charles H. Revson Foundation.

1. Weiner, S., Traub, W. & Lowenstam, H. A. (1983) in *Biomineralization and Biological Metal Accumulation*, eds. Westbroek, P. & de Jong, E. W. (Reidel, Dordrecht, The Netherlands), pp. 205–224.
2. Weiner, S. (1986) *CRC Crit. Rev. Biochem.* **20,** 365–408.
3. Addadi, L. & Weiner, S. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 4110–4114.
4. Addadi, L. & Weiner, S. (1986) *Mol. Cryst. Liq. Cryst.* **134,** 305–322.
5. Wada, K. (1980) in *The Mechanisms of Biomineralization in Animals and Plants*, eds. Omori, M. & Watabe, N. (Tokai Univ. Press, Tokyo), pp. 79–92.
6. Crenshaw, M. A. & Ristedt, H. (1975) *Biomineralization* **8,** 1–8.
7. Chen, C. C. & Boskey, A. L. (1985) *Calcif. Tissue Int.* **37,** 395–399.
8. Weinberg, P., Maroudas, A., Parker, K. H. & Winlove, C. P. (1986) *Curr. Adv. Skeletalogenesis* **3,** 121–125.
9. Thiele, H. & Awad, A. (1969) *J. Biomed. Mater. Res.* **3,** 431–444.
10. Maroudas, N. G. (1977) *J. Cell. Phys.* **90,** 511–519.
11. Katchalski, E. (1957) *Methods Enzymol.* **3,** 540–544.
12. Willis, H. A. & Zichy, V. J. I. (1978) in *Polymer Surfaces*, eds. Clark, D. T. & Feast, W. J. (Wiley, New York), pp. 287–307.
13. Worms, D. & Weiner, S. (1986) *J. Exp. Zool.* **237,** 11–20.
14. Hoare, D. G. & Koshland, D. E. (1967) *J. Biol. Chem.* **242,** 2447–2453.
15. Nagasawa, K., Inawe, Y. & Kamata, T. (1977) *Carbohydr. Res.* **58,** 47–55.
16. Gjerde, D. T., Fritz, J. S. & Schmuckler, G. (1979) *J. Chromatogr.* **186,** 509–519.
17. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
18. Tsay, T. & Veis, A. (1985) *Biochemistry* **24,** 6363–6369.
19. Holmes-Farley, S. R., Reamey, R. H., McCarthy, T. J., Deutch, J. & Whitesides, G. M. (1985) *Langmuir: ACS J. Surfaces Colloids* **1,** 725–740.
20. Greenfield, N. & Fasman, G. D. (1969) *Biochemistry* **8,** 4108–4115.
21. Parker, F. S. (1971) in *Application of Infrared Spectroscopy in Biochemistry, Biology and Medicine* (Plenum, New York), p. 601.
22. Birchall, J. D. & Davey, R. J. (1981) *J. Cryst. Growth* **54,** 323–329.
23. Jodaikin, A., Perl-Treves, D., Weiner, S., Termine, J. D. & Traub, W. (1986) *Int. J. Biol. Macromol.*, in press.
24. Fichtinger-Schepman, A., Kamerling, J. P., Versluis, C. & Vliegenthart, F. G. (1981) *Carbohydr. Res.* **93,** 105–123.
25. Weiner, S. & Erez, J. (1984) *J. Foraminiferal Res.* **14,** 206–212.
26. Simkiss, K. (1985) *Comp. Biochem. Physiol.* **16,** 427–435.
27. Watabe, N. & Pan, C. (1984) *Am. Zool.* **24,** 977–985.
28. Fisher, L. W., Termine, J. D., Dejter, S. W., Whitson, S. W., Yanagishita, M., Kimura, J. H., Hascall, V. C., Kleinman, H. K., Hassell, J. R. & Nilsson, B. (1983) *J. Biol. Chem.* **258,** 6588–6594.
29. Torchia, D. A., Hasson, M. A. & Hascall, V. C. (1977) *J. Biol. Chem.* **252,** 3617–3625.
30. Lee, S. L. & Veis, A. (1980) *Int. J. Pept. Protein Res.* **16,** 231–240.
31. Greenfield, E. M., Wilson, D. C. & Crenshaw, M. A. (1984) *Am. Zool.* **24,** 925–932.