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Tuning of Crystal Nucleation and Growth by Proteins: Molecular Interactions at Solid-Liquid Interfaces in Biomineralization*

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The mineralized tissues of a bivalve mollusk and a sea urchin are both composed of calcium carbonate crystals that are intimately associated with acidic glycoproteins. *In vitro* studies in which carboxylate-, carbonate- and phosphate-containing crystals are grown in the presence of partially purified acidic glycoproteins from these two tissues show that some of these macromolecules are able to interact specifically with certain crystal faces. Significantly all the affected crystal faces contain a common stereochemical motif. Interesting differences, however, were observed in the modes of interaction between the mollusk and sea urchin derived acidic glycoproteins. Only the former can induce oriented calcite nucleation *in vitro* and only the latter can interact from solution with specific calcite crystal faces. These differences are ascribed in part to the fact that the mollusk macromolecules are much more acidic than those from the sea urchin. Some of the acidic glycoproteins are also occluded inside the growing crystals. In the case of the sea urchin, and not of the mollusk, the proteins are preferentially located at specific crystal planes and their presence influences the mechanical properties of the crystal. A detailed study of these composite crystals by X-ray synchrotron radiation shows how the presence of the protein influences the crystal mosaicity. The interactions revealed by these studies follow well defined stereochemical rules, tuned by electrostatic forces. They, in turn, provide new insight into some of the basic underlying processes occurring in biomineralization.

Organisms from 55 different phyla are known to use some 60 different minerals in order to build solid phases that fulfill a large variety of functions¹. In many cases, the organisms are able to control the mineralization process at the molecular level. How this is accomplished and whether or not some common strategies are used, are some of the most important questions in the

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understanding of biomineralization. Furthermore, answers to these questions may provide important information to the materials sciences, as mineralized tissues often have mechanical material properties far superior to materials produced synthetically.

In many mineralized tissues crystal nucleation and growth occur in contact with a preformed matrix that includes acidic glycoproteins rich in carboxylate, sulfate and/or phosphate groups. In a number of systems, at least two sequential processes take place at the matrix/liquid interface during the formation of the crystalline phase. First acidic proteins adsorb and assemble on an insoluble scaffolding matrix to yield an hydrophilic charged surface. The ionic components of the crystal are then assembled on top of the resulting nucleating domains.

In a different type of biomineralized tissue, crystalline elements are formed within phospholipid vesicles, often composed of fused cell membranes. Mineralization is essentially an intracellular process. Acidic macromolecules are also found associated with the mineral, presumably adsorbed on the exposed surfaces of the developing crystals during growth.

It is our primary objective to understand the mechanisms of interaction between the macromolecules and the forming crystals, at the level of recognition that involves organized macromolecular surfaces and structured ionic crystal surfaces.

Two prototype systems have been used: the nacreous shell layer of the bivalve mollusk *Mytilus californianus*, and the skeletal plates and spines of the sea urchin *Paracentrotus lividus*. The aragonitic nacreous crystals are tablet shaped and have a uniform orientation with their *c* axes perpendicular to the associated layers of organic matrix macromolecules off which they grow². Some of the macromolecules that make up the core of this matrix layer are aligned at a molecular level with the aragonite *a* and *b* axes³. The morphology of the tablet-shaped aragonite crystals is quite unlike their inorganic counterparts. Furthermore the crystallographic planes at the surfaces of the crystal tablets are composed entirely of calcium ions or carbonate ions; an intrinsically unstable situation, which must be stabilized by a substrate.

The spines and skeletal plates of sea urchins are formed of magnesium-bearing calcite. Each whole element, spine or body plate, diffracts X-rays as a good quality single crystal of calcite. On the other hand, their spongy convoluted internal morphology and the conchoidal nature of their fracture surfaces are more akin to amorphous materials, and are certainly not typical of single crystals^{4,5}.

Both sea urchin skeletons and mollusk shells upon dissolution of the mineral, release into solution an assembly of acidic glycoproteins. Those extracted from the mollusk shell contain up to 50% aspartic and glutamic acid in their protein backbones, and have a large amount of covalently bound sulfated polysaccharides^{6,7}. The proteins extracted from the sea urchin skeleton are less acidic (Asp⁺ Glu = 25%), and their sugar and sulfate contents are also lower⁸. Both types of acidic glycoproteins partially assume the β -sheet conformation in solution in the presence of calcium.

We found that the acidic proteins from both the sea urchin and mollusk skeletal tissues are able to recognize specific faces of model crystals of calcium dicarboxylates displaying common stereochemical motifs^{9,10}. These con-

sist of rows of carboxylate groups emerging perpendicular to the interacting plane (Fig. 1a), such that their calcium complexation sphere can be optimally completed by a complementary domain of carboxylates of Asp and/or Glu on the protein β -sheet. Stereochemical requirements, thus, appear to be of paramount importance for these interactions to occur.

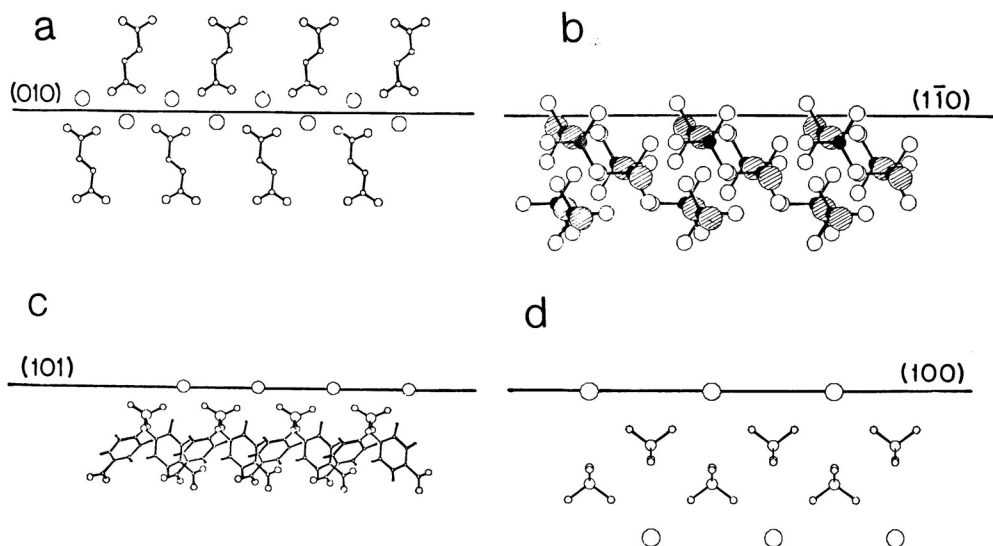


Figure 1. Isolated structural motifs emerging at the crystal surfaces selectively interacting with the acidic proteins. The structures are represented in an orientation perpendicular to the affected planes. a) Calcium fumarate (Ca = ●, C = ○, O = ○); b) Calcite (Ca = ●, C = ○, O = ○) the view has been rotated by 5° for easier understanding; c) Calcium bis-*p*-nitrophenylphosphate (Ca = ●, P = ○, C = ○, O = ○); d) Isolated motif emerging at the (100) face of apatite (Ca = ●, P = ○, O = ○).

A similar recognition pattern was partially identified in calcite crystals grown in the presence of sea urchin, but not mollusk proteins (Fig. 1b)¹¹ and was also identified on the interacting surfaces of crystals of calcium salts of organic phosphate diesters. The latter are being investigated as models for calcium phosphate containing biominerals (Fig. 1c)¹². The striking generality of these stereochemically controlled interactions drew our attention to the fact that the same stereochemical motif also exists on the (100) face of hydroxyapatite; the most developed face in enamel, dentin and bone crystals (Fig. 1d)¹³.

Calcite crystals grown in the presence of sea urchin acidic proteins are adsorbed and then occluded into the crystal in amounts of 0.02–0.1%. The resulting protein-containing crystals display a cleavage behaviour akin to that of the sea urchin skeletal elements and are very unlike the smooth cleavage typical of pure calcite crystals. These data strengthen the hypothesis that a possible function of the occluded proteins is to reinforce the biogenic calcite crystals by reducing their brittleness. In order to confirm this possibility, the influence of the protein on the texture of the composite crystalline material was characterized in synthetic protein containing crystals, and then

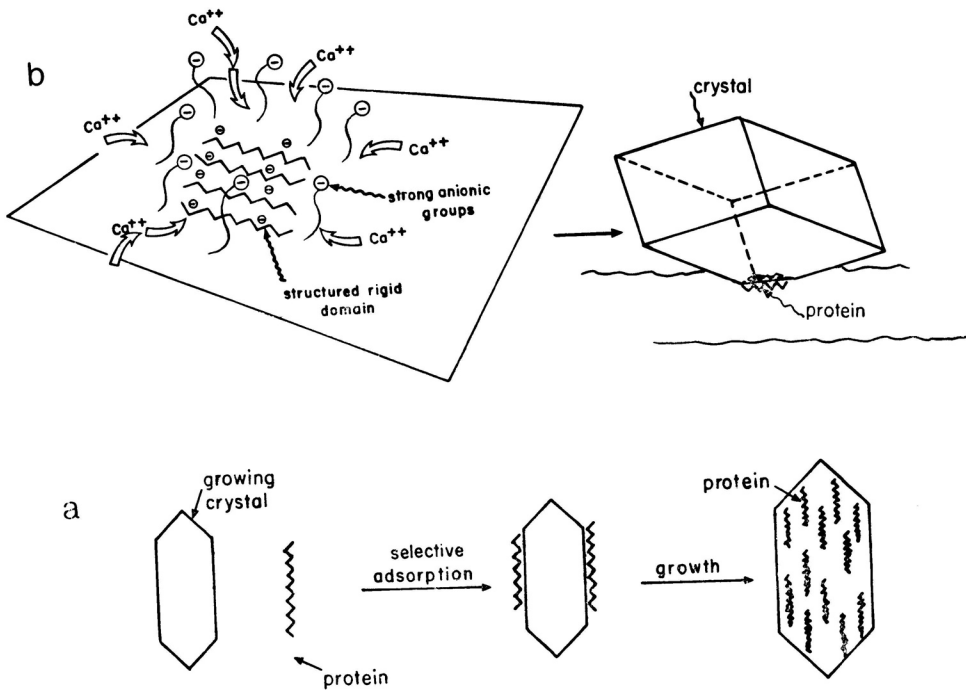


Figure 2. Schematic representation of the proposed mechanism for b) a nucleating protein adsorbed on a solid substrate; a) a 'modulating' protein being adsorbed on growing crystals from solution.

compared to the pure calcite crystals and to the biogenic ones from the sea urchin. For this purpose, diffraction peak lineshapes of the three types of crystals were examined with a highly collimated and monochromatic X-ray beam from synchrotron radiation¹⁴. The results show that the presence of small amounts of protein in both synthetic crystals and sea urchin skeletal elements does not drastically reduce the coherence length of perfect domains within the crystal as compared to those of pure calcite. On the other hand, introduction of protein into the crystal substantially increases the degree of misalignment between different perfect domains. This effect is particularly evident in the sea urchin spines. Occlusion of larger amounts of protein causes splitting of the diffraction peak in very sharp subpeaks, corresponding to a crystal formed of finite and quasiperfect mosaic blocks. The protein molecules must be located at the boundaries between these large blocks. The amount of protein however is, by orders of magnitude, smaller than what would be required in order to form continuous layers at the interface between blocks.

In contrast to the specific behaviour of the acidic glycoproteins extracted from the sea urchin, the presence in solution of analogous proteins extracted from the shell of the mollusk strongly inhibits the growth of crystals of calcite in all directions, in a way typical of the non-specific adsorption of a strong polyelectrolyte on charged calcite faces. On the other hand, we demonstrated that the *Mytilus* proteins are able, once adsorbed and rigidized on

a solid substrate, to induce *in vitro* oriented nucleation of calcite from a direction perpendicular to the *c* axis¹⁰. This occurs off uniformly charged calcium planes. We are thus confronted with a clear difference in the effect on calcite crystal growth, between acidic glycoproteins from the mineralized tissues of these two different organisms. Only the assemblage of mollusk acidic glycoproteins is able to induce (001) oriented nucleation of calcite, while only the sea urchin proteins are specifically adsorbed onto {110} planes of the growing crystals from solution. One possible explanation is in the much stronger ionic character of the mollusk shell nucleating proteins, due to their higher aspartic acid, sugar and especially sulfate content. When the molecules are in solution, they act as strong polyelectrolytes causing nonspecific adsorption on the highly charged surfaces of calcite. As the calcium dicarboxylate and phosphate diester surfaces are less charged, selectivity is still preserved on these substrates (Fig. 2a). In contrast, the less acidic surfaces of the sea urchin proteins do show selectivity for calcite crystal surfaces, but are not able to interact with calcium ions strongly enough to concentrate and organize the cations from solution and induce nucleation of new crystals. Nucleation from the mollusk proteins thus results from two factors cooperating together. The structurally disorganized sulfates create a flux of calcium ions towards the nucleation site and the carboxylates, regularly arranged in a protein β -sheet domain, provide the structural organization necessary for nucleation (Fig. 2b). Supporting evidence for this hypothesis was derived from experiments performed on an artificial system of a controlled chemical nature⁷. Increasingly sulfonated polystyrene surfaces, with and without adsorbed polypeptides, were used as substrates for crystallization. Oriented nucleation of calcite from the appropriate (001) face was achieved on films with a high surface concentration of sulfonates and with adsorbed β -sheet polyaspartate. The presence of only one of the two components, or of a polymer adsorbed in a different conformation, did not result in oriented crystallization.

A comparison between the influence of acidic glycoproteins from different calcified tissues on nucleation and growth of a series of different crystalline substrates begins, thus, to throw some light on the molecular interactions of this special type of organized solid-liquid interfaces. These interactions appear to follow well defined stereochemical rules, tuned by electrostatic forces. The observed generality of these rules *in vitro* may open the way to an improved understanding of some aspects of biomineralization.

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SAŽETAK

Kontrola nukleacije i rasta kristala proteinima: molekularno međudjelovanje na granici faza čvrsto/tekuće u biomineralizaciji

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Polazeći od činjenica da 55 raznih obitelji morskih organizama upotrebljavaju 60 raznih minerala za stvaranje čvrste faze i za ispunjavanje različitih funkcija, proučavana su mineralizirana tkiva školjaka i ježinaca, koji su oboje sastavljeni od kalcijevog karbonata i kiselih glikoproteina. In vitro proučavanja rasta karbo-ksilatnih, karbonatnih i fosfatnih kristala u prisutnosti djelomično pročišćenih glikoproteina iz tih organizama, pokazuju da organske molekule interagiraju sa specifičnim kristalnim plohamama. Sve takve plohe imaju neke zajedničke stereokemijske naznake. Proučavanja tih kristala primjenom rentgenskog sinhrotronskog zračenja dokazuju da prisutnost proteina prouzrokuje mozaički habit kristala. Interakcije koje su utvrđene ovim istraživanjima ukazuju na dobro definirana stereokemijska pravila kontrolirana elektrostatskim silama. Time se dobiva novi novi uvid u osnovne procese koji se dešavaju u biomineralizaciji.