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INTERACTIONS BETWEEN ACIDIC MACROMOLECULES AND STRUCTURED CRYSTAL SURFACES. STEREOCHEMISTRY AND BIOMINERALIZATION

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Abstract Some organisms are capable of exerting exquisite control over the crystals they form. Key components in this process are unusually acidic proteins (aspartic acid-rich). In this study we use a series of calcium dicarboxylic acid salts to investigate in vitro how these proteins are capable of interacting specifically with certain crystal faces. The property that characterizes the affected faces is the orientation of sets of carboxyl groups which are perpendicular to the plane of the face in well defined motifs. Calcite crystals grown under the same conditions were observed to nucleate off a stereochemically equivalent face and to be oriented with their c axes perpendicular to the substrate. We show that this is a result of the acidic proteins adsorbing onto the substrate and then inducing oriented nucleation. This study underscores the importance of these proteins in biological crystal growth and demonstrates some of the basic mechanisms involved.

Many characteristics of crystals depend on the interactions of the different structured crystal surfaces with their environment during nucleation and growth. Thus crystal shape, size and orientation are determined not only by the forces within the crystal lattice, but also by surface-environment interactions. Even crystal structure itself, at least with regard to the stabilization of one or another polymorph, may depend on the milieux in which the crystal grows. The influence of the environment on the crystal may manifest itself at very different levels, from the fairly non-specific, such as in solvent

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Incumbents of the Charles H. Revson[+] and Graham and Rhona Beck[○] Career Development Chairs

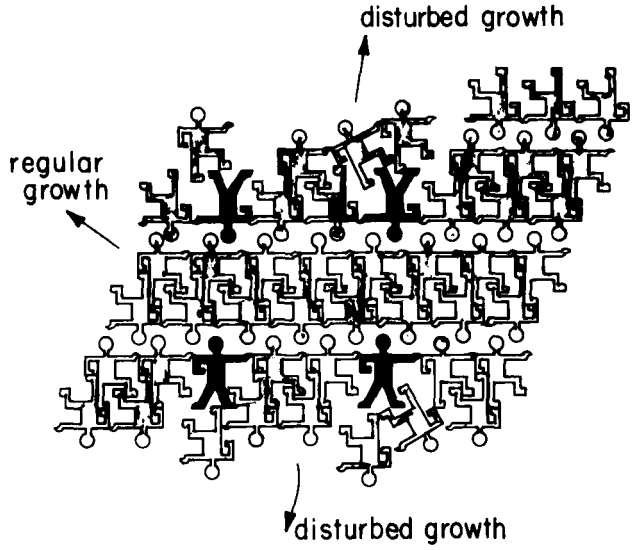
polarity or solubility effects, to the most highly specific as in epitaxial growth.

In controlled biological crystal growth one of the major strategies used by organisms to regulate crystal growth is also by means of surface-environment interactions. Many organisms are able to control crystal shape, size and orientation in order to construct complex structures such as bones, teeth or shells.

It is the primary aim of this study to understand some of the principles used by organisms in these processes, and in particular the mechanisms by which the biological macromolecules involved interact with the crystalline phase. Here we briefly summarise the relevant literature on 'stereochemical' crystal growth and biomineralization and then present the results of a novel approach to studying biological crystal growth. Part of this material was the subject of a recent publication.[1]

"Tailor made inhibitors": In the last few years the growth of organic crystals has been systematically studied by the solid state group at the Weizmann Institute, with a particular emphasis on the stereochemical correlation between growing crystal surfaces and crystal growth inhibitors ("tailor made" additives).[2] These are organic molecules, slightly modified with respect to the bulk component of the molecular crystal, which can selectively adsorb onto specific crystal surfaces. Adsorption of the foreign molecule causes drastic changes in the growth rate of the affected faces relative to the unaffected ones. Since crystal shape is determined by the relative rates of growth of the different faces, this eventually results in a change in crystal morphology and/or habit. By analysing the morphological changes, the affected and unaffected crystal faces can be differentiated. The structure of the affected faces can then be correlated with the molecular structure of the inhibitor.[3]

In this way, it was found that adsorption and subsequent growth inhibition occurred selectively on those faces at which the inhibitor occupies the site of a substrate molecule, such that the portion of the inhibitor which has a different molecular structure than the substrate molecules, points away from the crystal. It therefore does not substantially interfere with the regular crystal bond pattern. The modified moiety does, however, interfere with the deposition of the next molecular layer and as a result growth in that particular direction is dramatically reduced (Scheme 1).



Scheme 1

Once the basic mechanism of the effect was understood it was recognized that such morphological modifications are a very convenient tool for studying selective interactions at crystal surfaces. This approach was used to solve many different problems in the fields of enantiomer resolution,[4] direct assignment of absolute configuration of chiral molecules,[5] crystal morphology engineering,[6] crystal dissolution[7] and growth of polar crystals.[8] The knowledge acquired in these studies enables us now to investigate more complex problems such as solvent effect, crystal nucleation, epitaxial growth at structured surfaces[9] and, in the present study, biological crystallization.

Biogenic crystals are commonly formed by invertebrates and vertebrates where they are used predominantly for reinforcing skeletal structures. The crystals formed are usually of uniform size and shape, have constant well defined orientations and are

often quite different morphologically from their non-biological counterparts. Crystal polymorphism, when present, is genetically controlled. In some cases two polymorphs coexist within the same skeletal structure, but they are almost always spatially separated.[10]

A common mode of biological crystal growth involves the initial formation of a structural framework (the organic matrix) within which the crystals subsequently grow. Mollusk shells are particularly convenient systems for the study of matrix controlled crystallizations and have been the subject of many of our own investigations.[11,12] In some bivalves the shell is composed of an external layer of calcite (the prismatic layer) and an internal layer of aragonite (the nacreous layer). Both have a well defined microstructure, composed of arrays of hexagonal prisms in the calcitic layer, and a "brick wall" like structure in the aragonitic layer.[13] The crystals are each enveloped by the organic matrix composed primarily of proteins and polysaccharides.[14] In both layers the crystals are oriented with their c axes perpendicular to the shell. The axes of the aragonite in the nacreous layers of a variety of mollusks studied by us are aligned with the mean directions of the matrix proteins which adopt the β-sheet conformation.[15] Among the macromolecules that constitute the matrix in mollusk shells are a set of acidic, aspartic acid-rich proteins (representative amino acid composition Asp 30%, Glu 17%, Ser 10%, Gly 7%, Thr 7%).[16] These proteins are found predominantly on the matrix surfaces.[17] As they are, therefore, in close proximity to the crystal surface, they are generally thought to fulfill important functions in crystal growth regulation. A second class of acidic macromolecules present in the matrix are protein polysaccharides (proteoglycans?). Both classes bind calcium and in doing so undergo conformational changes[18] The aspartic acid-rich proteins adopt the β-sheet conformation. In this conformation the carboxylate side groups of the aspartic acid moieties all emerge in a planar array. Our working hypothesis is that crystal regulation is at least in part mediated by calcium ions shared between the carboxylate groups of the β-sheet proteins and the crystal surfaces.

In this study we have used the aspartic acid-rich proteins obtained from either the calcitic or the aragonitic layer of the bivalve Mytilus californianus.[12] We chose first to examine the manner in which these acidic matrix macromolecules interact with different structured surfaces of various calcium salts of organic dicarboxylic acids. Our ojective was to initally

understand the general stereochemical rules that govern these interactions. We then applied the derived rules to understand how these same proteins influence the growth of calcite in vitro.

Interactions Between Aspartic Acid-Rich Proteins and Ca Dicarboxylates: A Model System.

Crystals of calcium malonate dihydrate, calcium fumarate trihydrate, calcium tartrate tetrahydrate and calcium maleate monohydrate (cell dimensions in Table 1) were grown in the absence and in the presence of very small amounts of matrix macromolecules in solution (0.5-5μg protein/ml ~ 50-500 nM). Figs.1a, 2a, 3a and 4a show the measured morphologies of typical crystals of malonate, fumarate, maleate and tartrate, and Figs.1b, 2b, 3b and 4b show the morphologies of these crystals grown under the same conditions but in the presence of the proteins.

TABLE I: Cell dimensions of the calcium salts referred to in this study.

Compound	Space group	Cell dimensions a (Å)	b (Å)	c (Å)	(°)	Ref.
Ca-Malonate.$2H_2O$	$C2/m$	13.87	6.81	6.80	β 106	19
Ca-Fumarate.$3H_2O$	$Pna2_1$	6.62	17.63	6.97	-	20
Ca-Maleate.H_2O	$Pna2_1$	8.67	11.0	6.89	-	21
Ca-Tartrate.$4H_2O$	$P2_12_12_1$	9.27	10.63	9.66	-	22
Calcite	$R\bar{3}c$	4.99	4.99	17.06	γ 120	23

Changes in crystal morphology are clearly observed in Figs.1,2,3. The faces that have selectively adsorbed protein can be identified by their relative increase in morphological importance. These are {101} in the case of malonate, {010} for fumarate and {110} in the case of maleate. In the fourth system, Ca-tartrate, no specific morphological change can be detected (Figs.4a,b). Only at higher protein concentrations, a non-specific binding effect was manifested by a general loss of well defined crystal edges and shape, accompanied by the formation of holes and macroscopic steps in all crystal faces.

Figs.1c, 2c, and 3c show the structures of the affected salts of malonate (in a stereo view on the affected plane) fumarate and maleate (in a view perpendicular to the affected planes). Inspection of these structures clearly reveals a property common to all the systems, namely that at least one set of carboxylates emerges perpendicular to the plane of the face and complexes calcium in either or both of the main motifs shown in Scheme 2. In both motifs the carboxylates at the crystal surface are separated by a repeating distance of 6.6–6.9Å, which

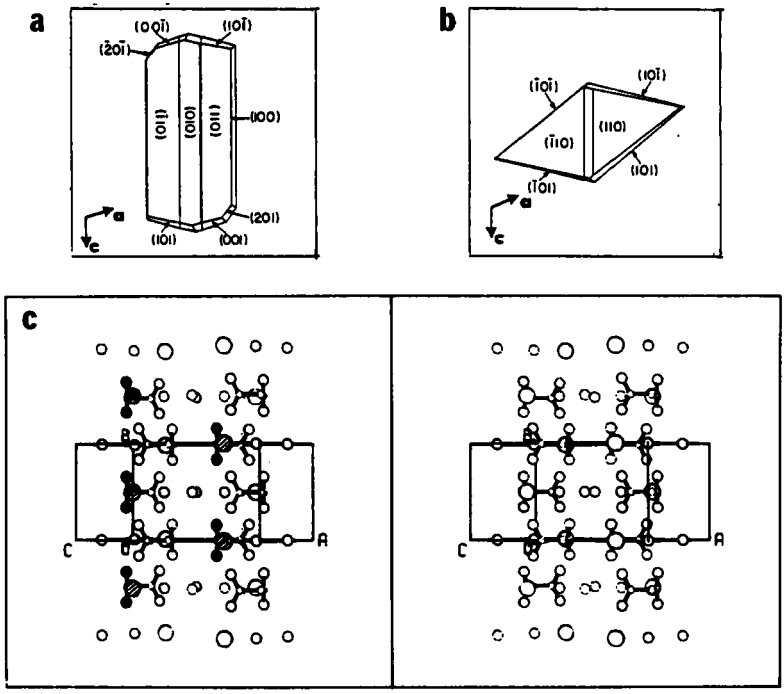
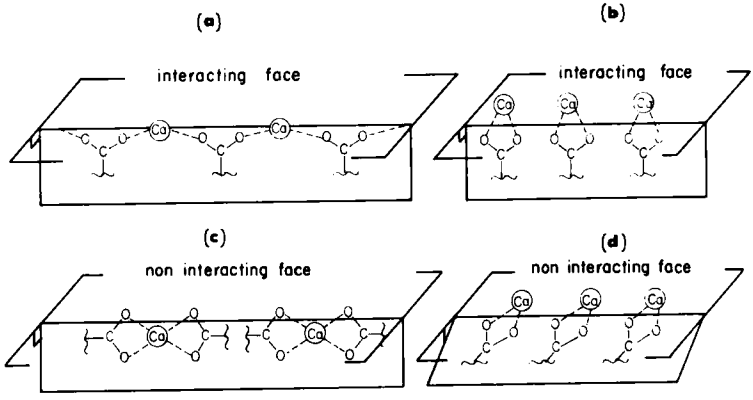


Fig.1 (a), (b) Measured morphology of typical crystals of malonate as seen along the b axis: (a) pure; (b) grown in the presence of proteins (0.5μg/ml); (c) stereoscopic projection of the structure of calcium malonate dihydrate on the affected (101) face. The large circles are Ca ions. The carboxylates perpendicular to the surface are blackened and the calcium atoms shadowed.

ideally matches the distance between side chains along the backbone of a β-sheet protein. The selectivity is not governed, however, by the repeating Ca-Ca distances alone. In this case any plane of the type, for example, (0hl) in fumarate or (h0l) in malonate would be an equally good candidate for adsorption. On the contrary, the importance of the orientation of the carboxylate groups, i.e. perpendicular to the face, is highlighted by the fact that in all the other faces of the crystals, unaffected by the protein, the carboxylates always emerge in a different orientation to the plane of the face. In particular, in the Ca-malonate system, the faces {201}, which are developed in the pure crystals, disappear in the affected ones in favour of the affected {101} faces (Figs.1a and b). The two planes are very similar in character, apart from an offset of $<30^{o}$ in the orientation of the carboxylates relative to the face. Furthermore, Ca-tartrate which is not specifically affected by the protein, does not possess any crystal planes with the required carboxylate orientation (Fig.4c).



Scheme 2

Protein selectivity is also not governed by the calcium density or cationic character of the face. This is particularly well demonstrated by the fact that in Ca-malonate the {100} face (Fig.5) has a high concentration of calcium ions and is indeed

the most developed face in the pure crystals. However, this face completely disappears in the affected crystals in favour of the less dense {101} faces.

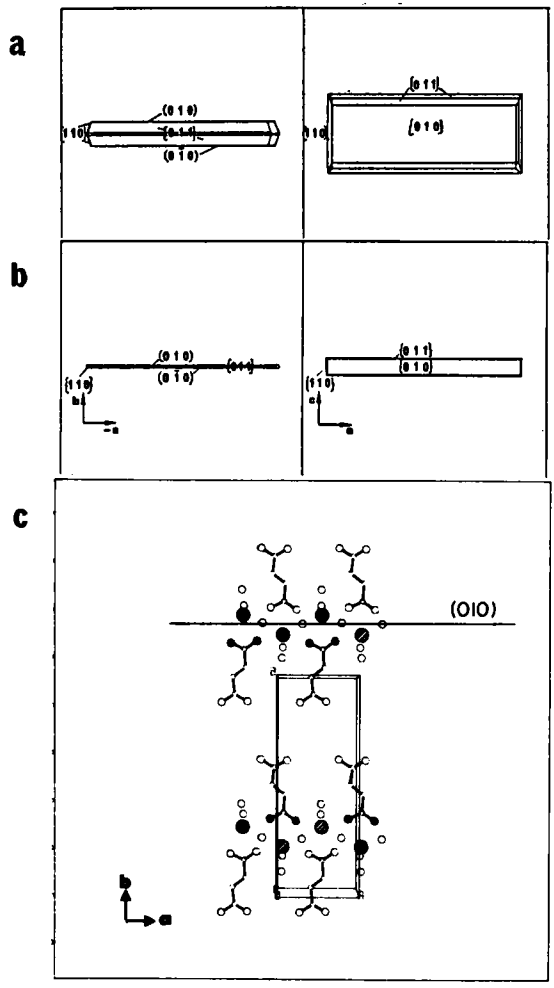


Fig.2 (a), (b) Measured morphology of typical crystals of fumarate as seen along the c and b axes: (a) pure; (b) grown in the presence of proteins (2μg/ml); (c) structure of calcium fumarate trihydrate viewed along the c axis. The affected (010) face is viewed edge-on. The calcium-carboxylate motifs are shadowed.

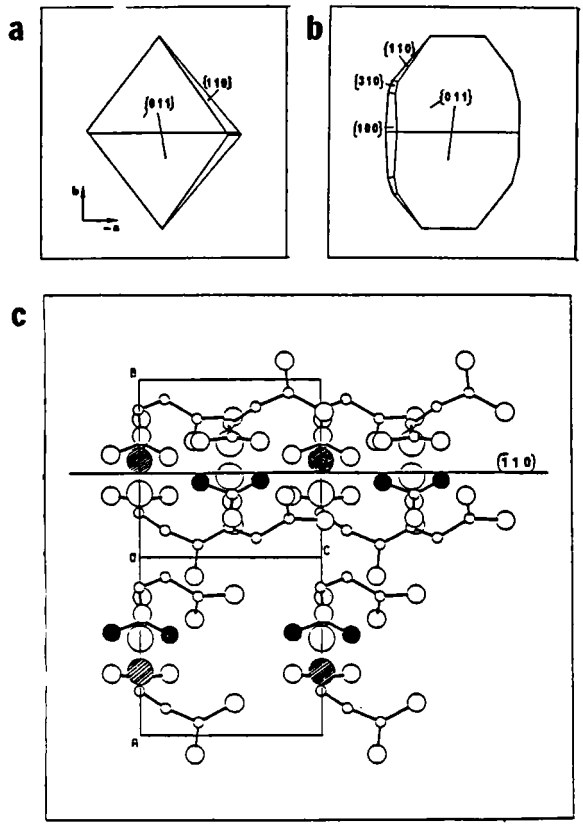


Fig.3 (a),(b) Measured morphology of typical crystals of maleate as seen along the c axis: (a) pure; (b) grown in the presence of proteins (0.5µg/ml). { } represent all the symmetry related faces; (c) structure of calcium maleate monohydrate viewed along line (-a +b). The affected (1̄10) face is viewed edge on. The calcium-carboxylate motifs are marked.

In order to assess the importance of cooperativity between binding ligands, as well as macromolecular conformation, we undertook some further model studies using synthetic polyaspartic and polyglutamic acids as inhibitors of the same

crystalline substrates. A first hint that the interactions between protein and crystal were cooperative in nature, was based on the very low amounts of inhibitors required to induce

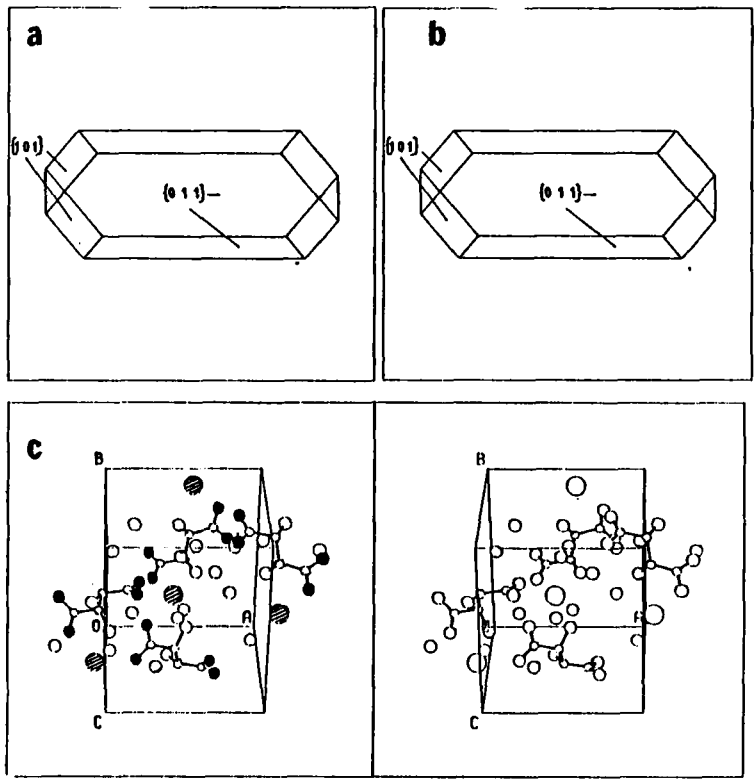


Fig.4 (a), (b) measured morphology of typical crystals of tartrate. (a) pure; (b) grown in the presence of proteins (0.5-5µg/ml); (c) stereoscopic projection of the structure of calcium tartrate tetrahydrate on the (011) face. Carboxylate oxygens are blackened and calcium atoms shadowed.

specific morphological changes. Aspartate and glutamate monomers do not affect crystal growth in the present systems, even at concentrations of approximately 10^4 higher than those required in the case of their polymerised counterparts. Note that the best low molecular weight tailor-made inhibitors are effective in the range of 1% wt/wt of substrate. Polyaspartate ($\overline{DP}$= 150) in solution adopts mainly the β-sheet conformation in the presence of Ca^{++}, as indicated by circular dichroism measurements. It indeed affects the growth of calcium maleate and fumarate in the same way as the aspartic acid-rich proteins.

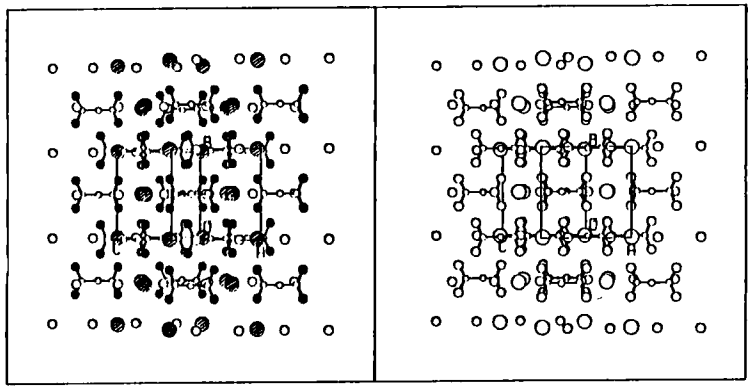


Fig.5 Stereoscopic projection of the calcium malonate dihydrate structure on the (100) face. Calcium atoms are shadowed and carboxylate oxygens are blackened.

In contrast, polyglutamate primarily adopts the random-coil conformation in the same solutions and has only a weak, non specific effect on crystal growth. It appears, therefore, that there is a common stereochemical effect in all systems, whereby partially rigid calcium loaded aspartic acid-rich matrix

proteins, which adopt the β-sheet conformation, can cooperatively bind only to those crystal faces characterised by motifs illustrated in Scheme 2A or B. It is only in this particular orientation that the side-chain carboxylates and calcium ions of the protein can occupy the lattice sites of the substrate ions over extended domains on the crystal surface.

Interactions Between Aspartic Acid-Rich Proteins and Calcite Crystals:

We now consider the implications of the stereochemical rule derived from the model systems to calcite, one of the common minerals formed by organisms and the mineral that interacts with these same proteins in the mollusk shells. The structure of calcite[23] (Fig.6) is characterised by having alternate layers of calcium and carbonate ions along the c axis. The planar

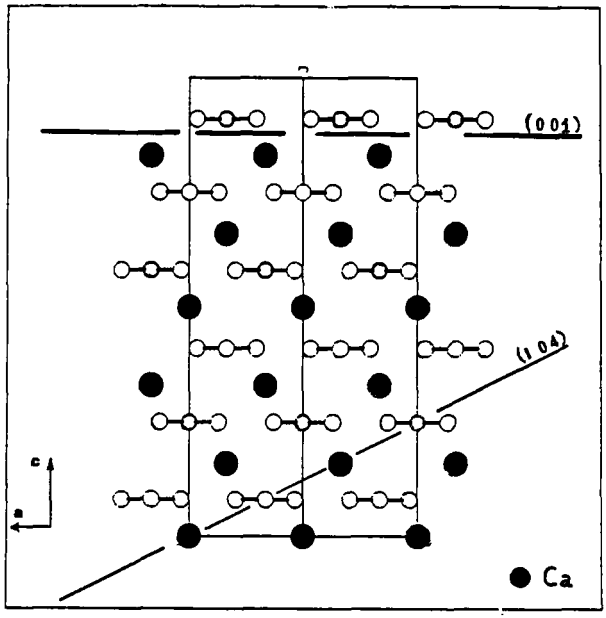


Fig.6 Structure of calcite viewed perpendicular to the c axis.

carbonate ions lie in the (001) plane. Of the three known polymorphs of calcium carbonate, two, calcite and aragonite,[23] have the same relative orientation of calcium and carbonate in parallel planes. The third polymorph, vaterite,[23] in which the carbonates are perpendicular to the calcium planes, is metastable and transforms spontaneously into the more stable forms. This indicates that for a carbonate, with 3 binding oxygens, the best approach to a calcium plane is parallel. In contrast, for carboxylates with two binding oxygens, we observed that the best approach is perpendicular to the plane. According to the stereochemical effect, the optimal plane for interaction between calcium-loaded aspartic acid-rich proteins and growing calcite should be {001}.

Calcite, grown by diffusion of $(NH_4)_2CO_3$ into $CaCl_2$ solutions, adopts the standard cleavage rhombohedron morphology,[2] in which the {104} faces are developed. The relative positions of the {001} faces with respect to the cleavage rhombohedron is shown in Fig.6 and 8b. Calcite crystals grown in the presence of small amounts ($< 0.5\mu g/ml$) of acidic proteins often show very small (001) faces which, curiously enough, are developed only at one vertex of the cleavage rhombohedron. Since (001) and $(00\bar{1})$ are symmetry related, both faces should develop as a result of the action of a kinetic inhibitor in solution, in a manner similar to those of the model systems. The development of one {001} face is compatible with a process of nucleation from the (001) plane. With no further interaction after nucleation, the crystals would then assume their cleavage rhombohedron morphology. We indeed observed that the affected crystals grew attached to the bottom of the glass vial, with the (001) face at the contact (Fig.7).

We could in fact unequivocally demonstrate[1] that the acidic proteins, once adsorbed on the rigid matrix are responsible for the nucleation of the calcite crystals from the (001) plane. This was achieved by growing calcite crystals in vials that were previously incubated with a protein solution, prior to the introduction of the crystallization solution. The effect was further confirmed by immunofluorescent labelling of the proteins associated with the (001) face of the nucleated crystals (Fig.8)[1].

We attribute the ability of the proteins to induce nucleation to their rigidity as a result of being adsorbed onto the substrate. Note that the same proteins when present in solution in relatively high concentration ($5\mu g/ml$), have a non-

specific inhibiting effect on the growth of calcite probably due to electrostatic attraction between the highly charged macromolecule and the crystal surface. This non-specific effect results in the formation of macrosteps on the crystal surface and in the general loss of well defined crystal morphology. It also masks any possible specific growth inhibition effect.

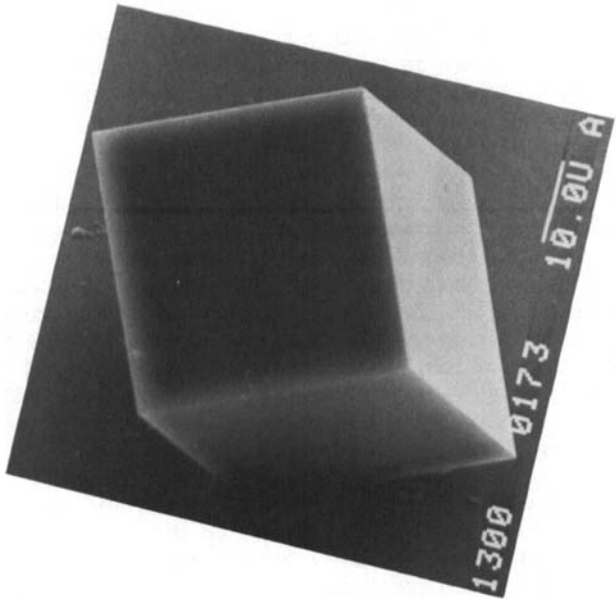


Fig.7 Scanning electron micrograph of calcite crystal nucleated on acidic proteins adsorbed on the surface of a glass vial, viewed at a tilt of 60°. The crystal is attached to the bottom through the small (001) face.

Discussion

The importance of acidic proteins of the kind used in this study can be inferred from their widespread distribution in mineralized tissues. They appear to be essential components in biological crystal regulation, as they are constituents of all organic matrices for which data are available.[24] In the few tissues where their locations are known, they are formed at the matrix surface in contact with the crystals.[11,17,25] Very little is known about the manner in which these proteins perform their functions *in vivo*. The present study does, however, raise

the possibility of partially reconstructing matrix functions from an analysis of the orientation and of the expressed faces of the biogenic crystals. For calcite and aragonite (which has an almost identical calcium layer in the (001) plane), if the c axis of the mineral is oriented perpendicular to the matrix, this would strongly suggest that β-sheet acidic proteins are actively involved in regulating crystal nucleation and growth.

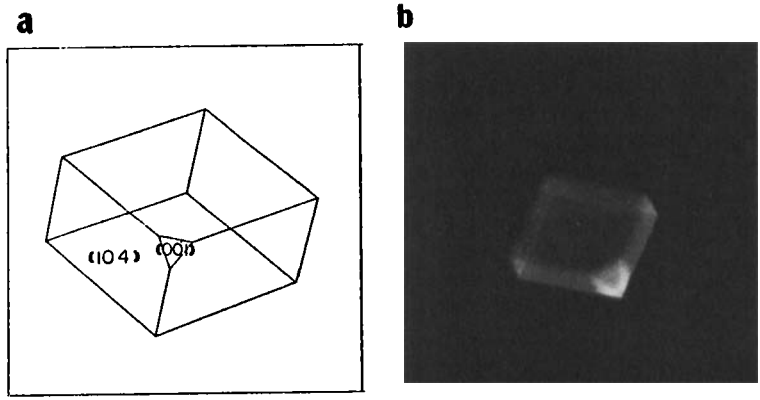


Fig.8 (a) Computer drawn simulation of a calcite crystal in which one of the {001} faces is developed at the vertex of the cleavage rhombohedron; (b) immunofluorescent stained calcite crystal demonstrating the presence of aspartic acid-rich protein on the (001) face.

Calcite and aragonite are common biogenic minerals formed by many different organisms,[10] and in the overwhelming majority of cases they are indeed found to develop with their c axes perpendicular to the organic substrate off which they grow.[26]

In terms of the stereochemical effect demonstrated in this study, we would not necessarily expect the crystals nucleated on the β-sheet protein to have their a and b axes aligned in a specific manner with the polypeptide chains of the protein (as would be expected for true epitaxial growth). This spatial relation however, has been demonstrated at least in one mineralized tissue, the aragonite bearing nacreous layer of

mollusks,[15] suggesting that some organisms are able to exercise far more control over crystal nucleation than is explained from the stereochemical rules.

The relevance of this stereochemical effect to biogenic minerals other than carbonates is not yet known. We do note that the {100} face of hydroxyapatite, a commonly expressed face in the crystals of bones and teeth does contain a chain motif.[1] This is analogous to the carboxyl structure in the plane of the affected faces of the calcium dicarboxylic acid salts.

This study also has a number of interesting implications for materials science. The ability of the acidic matrix proteins to recognize certain crystal faces and not others, or to induce oriented crystal growth, raises the possibility of designing polymers to perform the same functions and to use these polymers to fashion crystals with advantageous properties. The same principle can be extended to modifying surfaces such that they will be able to induce controlled crystal nucleation. We have initiated a series of experiments along these lines in which polystyrene films are sulfonated to varying degrees and to which polyaspartic acid is adsorbed.[27] We observed that oriented calcite crystals (i.e. nucleated from the (001) face) can form on this substrate under conditions in which the polystyrene film alone or the adsorbed polyaspartic acid alone are not able to induce oriented crystal growth. On the other hand, polyacrylate or even polyglutamate when adsorbed onto the same sulphonated film, are not able to induce oriented nucleation. This indicates that the two kinds of groups interacting with calcium, i.e. sulphonates which have a strong concentrating effect, and carboxylates in the correct organisation, act cooperatively. We are presently investigating the nature of the cooperative effect, and its possible implications for the mechanism of biomineralisation in general and of mollusk shells in particular.

ACKNOWLEDGEMENTS

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