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An AFM investigation of the interaction of chiral amino acids with the {104} face of calcite

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Abstract

In biomineralized tissue, Nature often uses a single crystal system to form tools with widely varied form and functionality. To accomplish this, organisms have developed methods to deterministically modify and control crystal habit, commonly creating shapes with lower symmetry than is possessed by the pure crystal. In this paper we use atomic force microscopy to investigate the effect of chiral amino acids on calcite growth. We show that the atomic steps and resultant macroscopic shape exhibit a lower symmetry that reflects the chirality of the amino acid. We use this result to constrain the possible stereospecific binding sites. We argue that the change in morphology is not due to the incorporation of the amino acid, but rather that it acts like a surfactant changing the energetics of the interface. These results suggest that the conventional paradigm for understanding the geometrical and chemical aspects of biomineralization in terms of stereochemical recognition should be expanded to capture the energetic controls that determine the mechanisms of mineral modification by biomolecules.

Introduction

Through the process of biomineralization, living organisms use selective introduction of peptides and proteins(1-3) to deterministically modify crystal nucleation(4), growth kinetics(5), surface morphologies (6-8), and facet stabilities(4, 6-8). As a result, they are able to generate nanophase materials, single-crystals, and multi-layer composites for a diverse set of biological functions(2, 5, 7). This phenomenon provides an elegant example of self-organization in complex molecular systems and demonstrates the potential for biomimetic processing in materials science. However, the fundamental physical controls on biomineral formation remain poorly understood.

Taking calcite with acidic amino acids as a simple model system, we used *in-situ* atomic force microscopy (AFM), nucleation and growth of bulk crystals on self-assembled monolayers (SAM), and surface spectroscopy to examine the energetic factors controlling the modification of calcite growth. Our results demonstrate that the introduction of chiral amino acids leads to the emergence of asymmetric crystal structures that reflect this chirality. Moreover, they show that the organic fraction acts as a surfactant, binding to step edges on the calcite surface and altering the interfacial energetics. This conclusion suggests that while the concept of stereochemical recognition commonly used to describe the interaction of organic growth modifiers with biomineral surfaces may account for the structural and chemical relationships present during mineralization, the mechanism of crystal modification is better understood by considering surfactant-mediated changes to the energy landscape seen by the adsorbing and desorbing species at the surface of the mineral.

A number of investigations have shown that the soluble organic fraction associated with mineralizing parts of organisms plays a primary role in controlling carbonate biomineral formation. For example, Belcher et al. (3) showed that exposing growing CaCO₃ crystals alternately to solutions containing polyanionic proteins associated with the aragonitic and calcitic layers of abalone shells led to sequential switching of the crystal structure of the newly grown material between that of aragonite and calcite. In general, the soluble organic fraction of carbonate

biominerals is characterized by the common presence of acidic amino acid residues, particularly aspartic acid (Asp)(9-11). Carbonates exposed to different acidic polyamino acids have been shown to exhibit modified crystal structures and/or habits(6, 12, 13). Furthermore, Wierzbicki et al.(14) found that polyaspartate molecules (Asp_{20}) bind preferentially to specific calcite surfaces and that calculations of Asp_{15} interactions with calcite planes predicted large binding energies for well-defined orientations. Consequently, the calcium carbonate-amino acid system provides an attractive model for investigating biomineralization processes.

Atomic step structure

Under the conditions used in this work, growth of calcite in pure solution resulted in expression of bulk rhombohedral crystals with six crystallographically equivalent $\{104\}$ facets (Fig. 1a). This simple form reflects the atomic scale controls on symmetry: the terraces of the growth hillock, shown in Fig. 1b, are comprised of $\{104\}$ faces while the step risers are produced by the intersection of those faces with the adjacent $\{104\}$ planes (Fig. 1c)(15-17). This produces two steps that form an acute angle with respect to the $\{104\}$ cleavage plane and two steps that form an obtuse angle with respect to the that plane (Fig. 1d). The two obtuse steps are related through a glide-plane symmetry element as are the two acute steps(17).

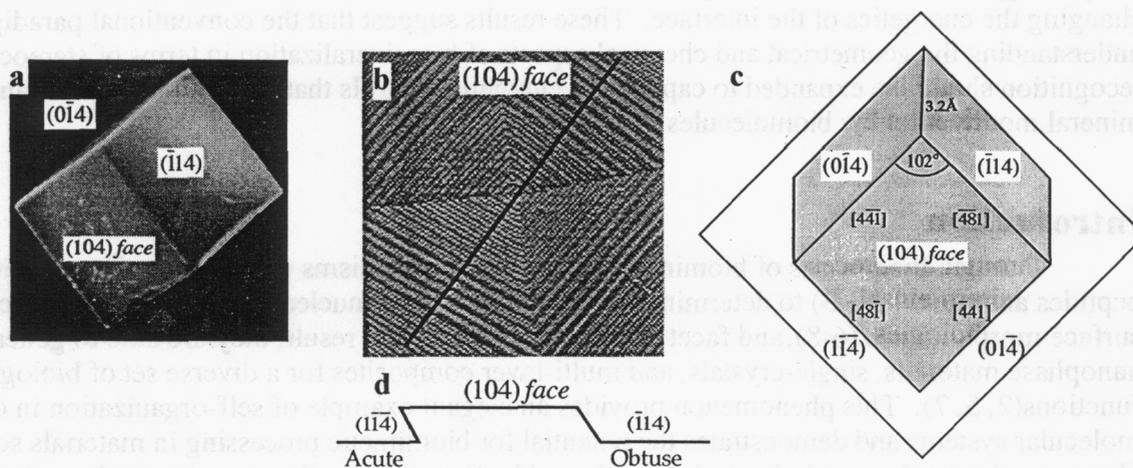


Figure 1 - (a) SEM micrograph of a typical calcite rhombohedron grown in pure solution. (b) 20x20µm AFM image of a growth hillock on a calcite $\{104\}$ face in pure solution showing the typical rhombohedral form where each of the four steps is bounded by adjacent $\{104\}$ microfacets. (c) Schematic of a calcite structure showing the relationships between the facet and step directions. (d) Schematic cross section of an island taken along the direction of the solid line in (b) showing the orientations of the acute and obtuse steps.

Upon addition of glycine, an achiral amino acid, the two acute steps became curved, losing their well-defined facet directions (Fig.22). The two obtuse steps were unaffected and, overall, the growth hillock remained symmetric about the glide plane. In a similar manner, upon addition of Asp-bearing solution, the two acute steps became curved and the two obtuse steps remained unperturbed. However, with the addition of pure D- or L-Asp, the symmetry about the glide plane was broken and the resultant morphology depended upon the chirality of the aspartic acid (Fig. 2c and 2d). Moreover, growth hillocks formed in the presence of D- and L- forms of Asp were mirror images of one another. Growth of calcite in serine and glutamic acid also resulted in loss of symmetry about the glide plane (Fig. 2e and 2f). During dissolution of pure calcite in Asp-bearing solution, we again observed enantiomer specific facet expression (Fig. 3a-3d). In fact, for all

cases in which we added chiral amino acids, we observed a reduction in symmetry both during growth and dissolution. A key result of this study is that the binding of amino acid enantiomers to calcite steps reduces the symmetry of the composite system, with the D and L forms affecting the complimentary steps on opposite sides of the calcite glide plane in an identical manner. Thus, the chiral nature of the amino acid binding breaks the crystal symmetry of the calcite growth hillock and leads to the emergence of a chiral configuration of the growth steps. Further, the fact that these effects occur during dissolution when none of the additive has been introduced into the bulk crystal indicates that these effects are strictly the result of a surface interaction.

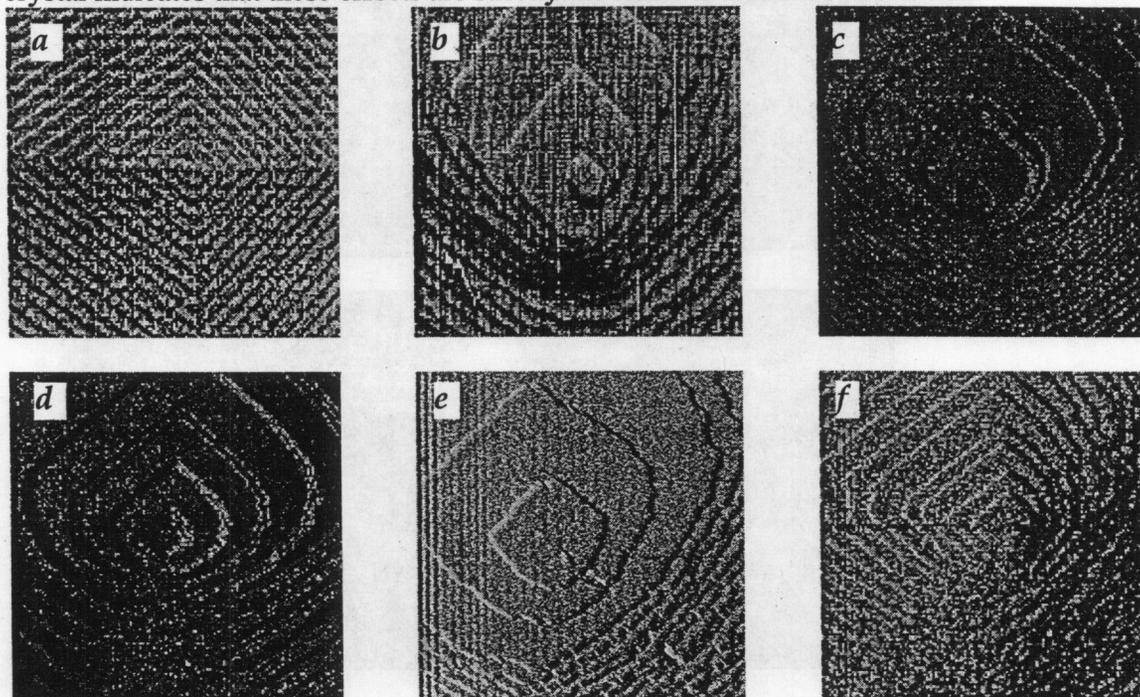


Figure 2 - AFM images showing effect of amino acids on growth hillock geometry. (a– c) show growth hillocks in (a) pure solution, and following addition of (b) 0.01M glycine, an achiral acid (c) 0.01M D-asp, and (d) 0.01M L-asp, (e) 0.01M L-serine and (f) 0.01M L-glutamic acid. All images are shown in same orientation as in Fig. 1c with two obtuse steps at top of image. Image sizes are: (a) 4x4 μm , (b) 3x3 μm , (c) 15x15 μm , and (d) 15x15 μm , (e) 3x3 μm , and (f) 4x4 μm .

Macroscopic crystal shape

To investigate how the observed changes in the atomic-scale surface structure translate into alterations in the overall shape of the bulk crystal, we nucleated calcite crystals from supersaturated solutions on patterned SAMs of alkanethiols on gold using a method similar to that described by Aizenberg et al. (18). As Fig. 4 shows, several new features emerged during growth of these crystals in the presence in Asp. First, a new set of facets was expressed that lie approximately parallel to the $\langle 001 \rangle$ axis of pure calcite and map to the $\{hk0\}$ family of planes. A trio of $\{10\bar{4}\}$ facets capped the crystals. In addition, the crystals were elongated along the $\langle 001 \rangle$ direction implying that the growth rate was anisotropic. (Similar expression of $\{hk0\}$ -like faces was observed for the growth of calcite in presence of the acidic fraction of proteins found in association with sea urchin spine skeletons(19).) Second, the most striking feature of the crystals grown in the presence of aspartic acid was the reduction in symmetry of the $\{10\bar{4}\}$ caps. Furthermore, bulk

crystals formed in presence of the two enantiomers were related to each other by mirror symmetry just as past experiments have shown macroscopic etch pits to be related in this manner(20).

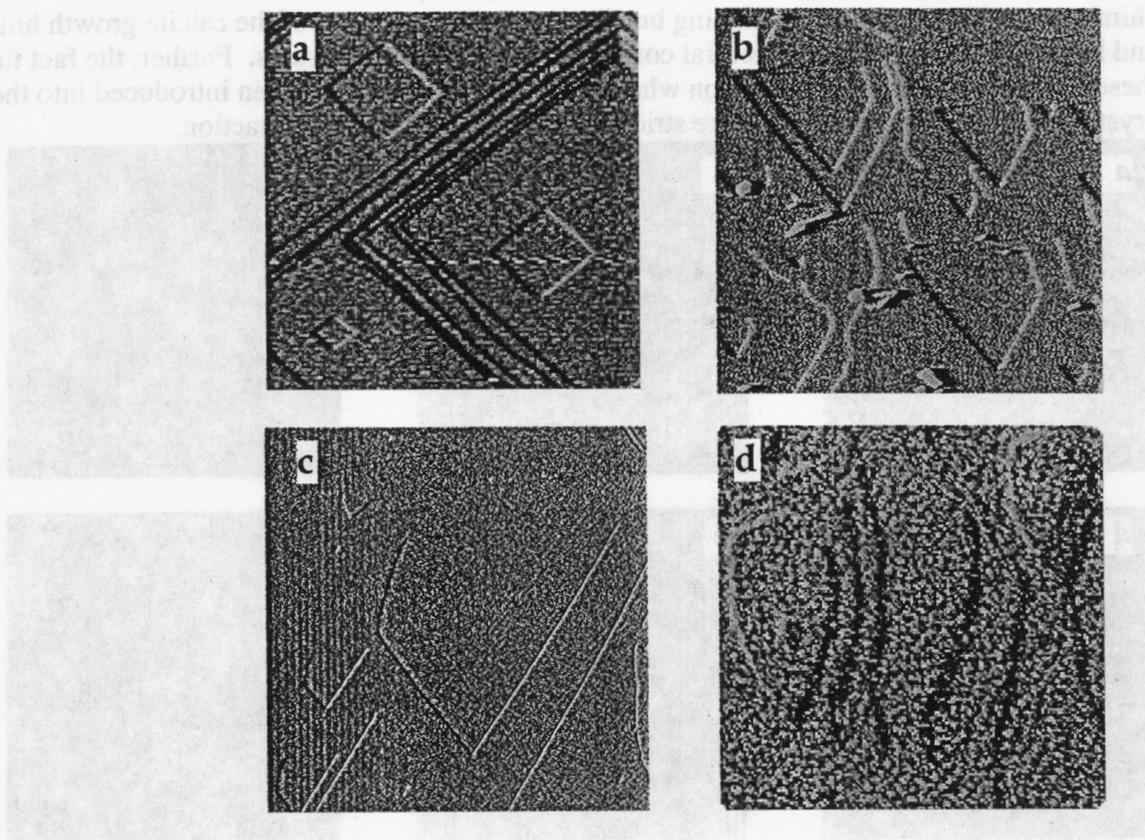
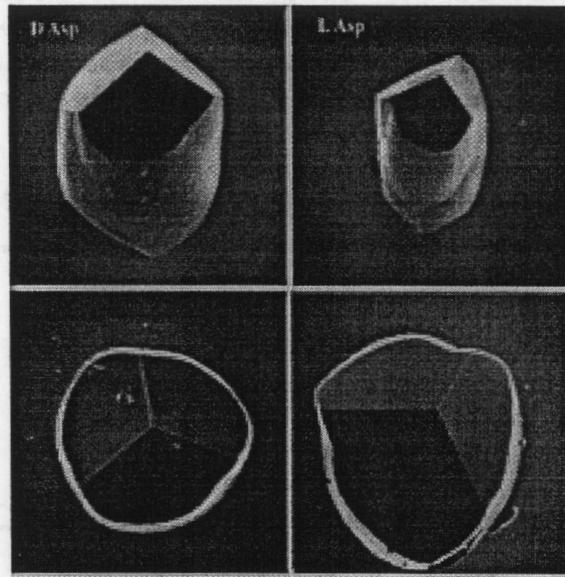


Figure 3 – AFM images of dissolution pits at high undersaturation for (a) pure solution, and 0.01M solutions of (b) D-asp, (c) L-Asp, and (d) a racemic mixture of L- and D-asp. All images are shown in same orientation as in Fig. 2 but note step directions in pits are the reverse of those on hillocks. Image sizes are: (a) 4x4 μm , (b) 5x5 μm , (c) 15x15 μm ., and (d) 3x3 μm .

SEM images taken down the $\langle 001 \rangle$ axis show that the three-fold symmetry of the crystals about the c-axis was generally conserved. Remarkably, the shape of the cap facets was almost exactly the same as the shape exhibited by calcite atomic steps at growth hillocks after the introduction of aspartic acid into the growth solution (Fig. 2). The obtuse steps unaffected by the aspartic acid formed the straight ridges connecting the cap facets and the curved acute steps formed the rounded base of the cap. These observations demonstrate that changes in the shape of the atomic steps resulted in the direct modification of the macroscopic shape of the crystals and led to the appearance of the new family of faces approximately parallel to the c-axis. Moreover, breaking the symmetry of the atomic steps translated to a reduction in overall crystal symmetry and drove the emergence of the chiral shapes, suggesting a potential mechanism by which living organisms create chirality in biomineralized structures.

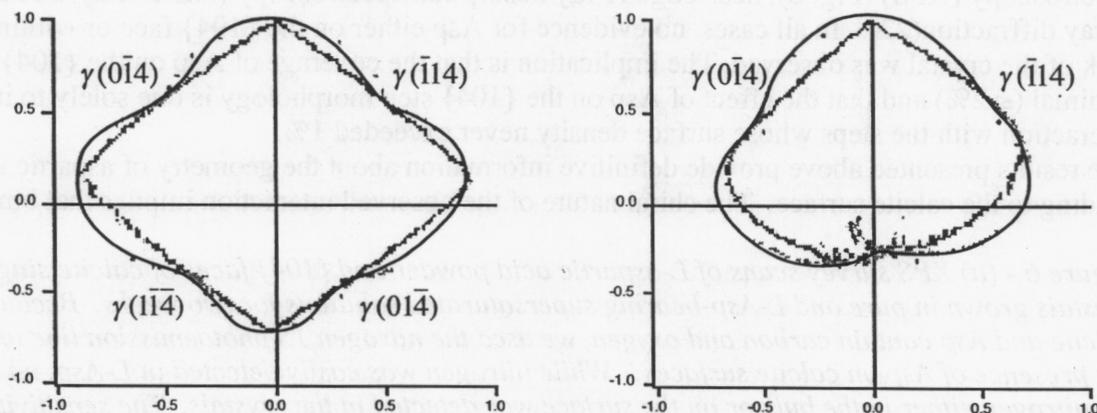
The similarity of the atomic scale morphology of the growth hillocks on the $\{104\}$ facet — particularly the first turn of the growth spiral — to that of the bulk crystal suggests that the facet exhibits an equilibrium geometry. Even hillocks grown in solutions with near equilibrium CaCO_3 concentrations displayed this modified shape, reinforcing this conclusion. Consequently we used

the facet shape to construct a polar plot of the step edge energy (i.e., a 2D Wulff plot) on the $\{104\}$ face by established methods(21). Although the highly rigid step morphology in the pure system provided us with little information about the shape of the energy surface, from previous measurements of the free energy associated with formation of a growth spiral, we knew that the free energy per unit length along the curved portions of the steps near the hillock corners is much



larger than that of the straight portions(15). As a result, the Wulff plot derived from the pure calcite

Figure 4 - SEM micrographs of calcite crystals nucleated on the COOH-terminated regions of alkanethiol SAM and grown in the presence of 0.01M Asp. All crystals nucleated on $\{001\}$ facets and exhibited a columnar morphology. The trio of facets forming the pointed cap of each column are the $\{104\}$ facets and the straight ridges separating the facets correspond to the obtuse steps on those facets. The shape and chirality of each facet matches that of the growth hillocks shown in Figs. 2c and 2d. The curved surfaces that form the barrel of each column have general $\{hk0\}$ orientations.



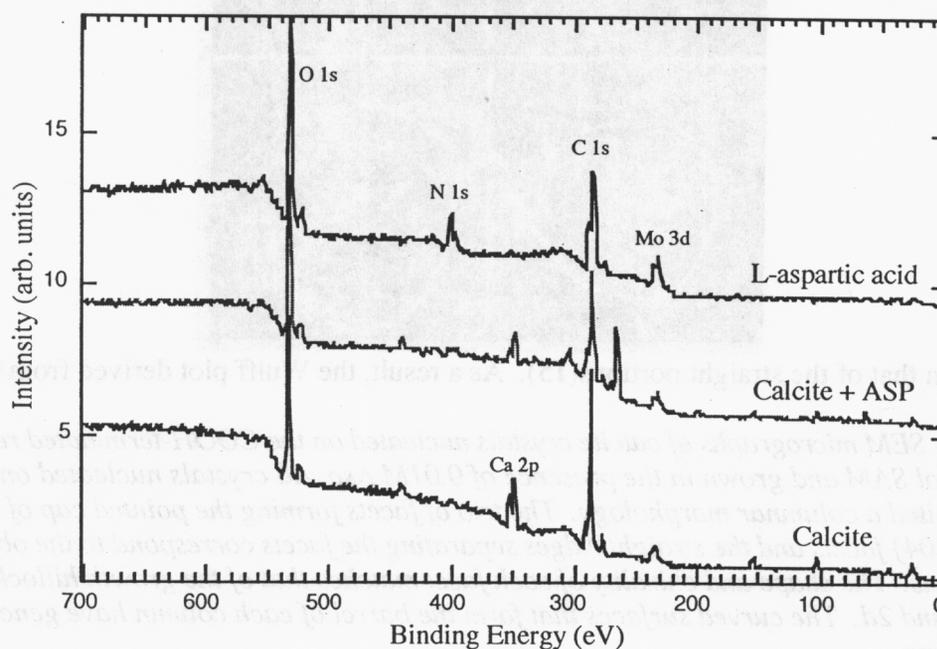
growth-form exhibits deep minima along the four step directions as illustrated in Fig. 5a. In contrast, in the presence of Asp, the hillock was much more isotropic near the three corners involving the acute steps. As Fig. 5b shows, in the resulting Wulff plot for the Asp-bearing

system, the minima associated with the two acute step edges were eliminated, demonstrating that the energetic cost of creating curvature — and therefore kink sites — was significantly diminished. In addition, a new minimum appeared near the intersection of the glide plane with the two acute step edges, but its position switches from the right to the left side of the glide plane when the amino acid is changed from D-Asp to L-Asp. The appearance of this new minimum indicates that the addition of Asp generates a new low-energy orientation for the step.

Figure 5 - Orientational dependence of step edge energy (2D Wulff plot) in the {104} plane based on equilibrium crystal shapes for crystals grown in (a) pure solution and (b) 0.01M D-Asp-bearing solution. The 2D Wulff plot for L-Asp-bearing solutions is the mirror image of 4b.

Adsorbate-surface geometry

To determine the structural relationship between aspartic acid and the {104} calcite surface,



we performed a set of surface-sensitive spectroscopic measurements including X-ray photoelectron spectroscopy (XPS) (Fig. 6), near-edge X-ray absorption spectroscopy (NEXAFS), and surface X-ray diffraction(22). In all cases, no evidence for Asp either on the {104} face or within the bulk of the crystal was observed. The implication is that the coverage of Asp on the {104} face is minimal ($\leq 2\%$) and that the effect of Asp on the {104} step morphology is due solely to its interaction with the steps whose surface density never exceeded 1%.

The results presented above provide definitive information about the geometry of aspartic acid binding to the calcite surface. The chiral nature of the observed interaction implies that binding

Figure 6 - (a) XPS survey scans of L-aspartic acid powder and {104} faces of calcite single crystals grown in pure and L-Asp-bearing supersaturated solutions for two weeks. Because both calcite and Asp contain carbon and oxygen, we used the nitrogen 1S photoemission line to detect the presence of Asp on calcite surfaces. While nitrogen was easily detected in L-Asp, no evidence for nitrogen either in the bulk or on the surface was detected in the crystals. The sensitivity of the XPS measurements was such that a surface coverage in excess of 2% should have been detectable.

must span the chiral center of the amino acid. In addition, if the binding was localized at only one site on the calcite surface, an identical position could be found on the opposite side of the glide

plane that, by definition, exhibits mirror symmetry. Consequently, an individual amino acid molecule must affect at least two sites on the calcite face to exhibit chiral binding. Finally, lack of evidence for Asp on the {104} faces implies that the interaction only occurs at the step edges and the resulting expression of near-{hk0} faces implies that the structure of those step edges is modified.

Mann et al.(7) previously proposed similar bidentate substitutional bonding to calcite {hk0} faces for molecules which have two carboxyl groups such as aspartic acid, glutamic acid and maleic acid. These authors presented schematic illustrations of potential binding geometries on the {110} faces. Our results are in general agreement with this proposal with one important difference: AFM results clearly show that the surface grows on single steps even in the presence of the amino acids. Therefore, we are forced to conclude that acidic amino acids do not bind onto a single {hk0} crystallographic plane during growth, but rather they bind at the steps edges. Furthermore, because we require two-site binding, the Asp-calcite complex must involve sites on both the step riser and the terrace.

Discussion

The macroscopic structure of crystals within biomineralized tissue is, to a large extent, responsible for the mechanical properties and function of the tissue. A living organism has no other means to control crystallization except by modifying the molecular level structure via either surface binding of proteins or changes in the local chemical environment at the growing surface. Therefore, understanding the relationship between growth modifiers, atomic step structure and overall modifications in crystal morphology, as well as the energetic and kinetic basis for those modifications is a key to understanding mechanisms of biomineralization. The results presented here demonstrate that site-specific binding of chiral amino acid residues leads to chiral modifications at atomic length scales that are manifest at macroscopic length scales and that those changes result from a surface interaction that modifies the interfacial energetics.

This interpretation serves to expand the conventional paradigm that defines current views on biomineralization and is often captured in the term "stereochemical recognition" (1, 4, 6, 7, 11, 23). As is implied by the term, this paradigm emphasizes geometrical and chemical incentives to binding. However, this description begs the question of mechanism. The nucleation of biominerals in specific locations with well-defined orientations as well as their growth into highly modified shapes are both governed by equilibrium thermodynamic and kinetic factors. Our investigation indicates that, at least in the case of acidic amino acids on calcite, the addition of the organic growth modifier alters the energy landscape seen by adsorbing and desorbing species at the surface of the mineral, and results in changes to the rates of attachment and detachment and the lowest energy configuration of the interface. In other words, while stereochemical recognition may describe the structural and chemical relationships present during mineralization, the mechanism of crystal modification is better understood by considering surfactant-mediated changes to the interfacial energies.

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