Molecular modulation of calcium oxalate crystallization

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Am J Physiol Renal Physiol 291: F1123–F1132, 2006; doi:10.1152/ajprenal.00136.2006.—Calcium oxalate monohydrate (COM) is the primary constituent of the majority of renal stones. Osteopontin (OPN), an aspartic acid-rich urinary protein, and citrate, a much smaller molecule, are potent inhibitors of COM crystallization at levels present in normal urine. Current concepts of the role of site-specific interactions in crystallization derived from studies of biomimetic mineralization are reviewed to provide a context for understanding modulation of COM growth at a molecular level. Results from in situ atomic force microscopy (AFM) analyses of the effects of citrate and OPN on growth verified the critical role of site-specific interactions between these growth modulators and individual steps on COM crystal surfaces. Molecular modeling investigations of interactions of citrate with steps and faces on COM crystal surfaces provided links between the stereochemistry of interaction and the binding energy levels that underlie mechanisms of growth modification and changes in overall crystal morphology. The combination of in situ AFM and molecular modeling provides new knowledge that will aid rationale design of therapeutic agents for inhibition of stone formation.

citrate; osteopontin; atomic force microscopy; biomimelarization; kidney stone

Calcium oxalate monohydrate (COM) is the primary constituent of the majority of stones formed in the urinary system of patients with urolithiasis (11). Formation of stones within the urinary tract is a complex process influenced by multiple factors. Although normal urine is frequently supersaturated with respect to calcium and oxalate (CaOx), most humans do not form stones. Typically, any crystals formed are rapidly passed before achieving a size sufficient for retention. Increased quantities of calcium and/or oxalate are excreted by many COM stone formers (22, 57). However, increased supersaturation alone does not account for the incidence of stone disease (5) because other factors also influence the formation and growth of stones. These factors include the rate of urinary flow that sweeps crystals downstream and the effects of other urinary constituents on rates of crystal nucleation, rates of crystal growth, and the extent of aggregation of crystals and crystal binding to cells. Although the relative contribution of each is unclear, a number of inhibitors of COM crystallization in normal urine that impact a number of these factors have been identified (3, 18, 25, 42). Such inhibitors raise the concentrations of calcium and oxalate required for spontaneous formation of new crystals and decrease crystal growth, aggregation, and binding to renal cells. Furthermore, COM crystallization in vitro is significantly more inhibited by urinary proteins from normal subjects than by urinary proteins from stone formers (4, 58). Although presumed to be protective, the precise roles of inhibitors of CaOx crystallization have not been defined. The majority of the inhibition of COM crystal growth observed with in vitro assays of normal urine is due to the presence of protein macromolecules (18, 47). Such protective effects of inhibitors may be especially important during times of physiologically increased calcium excretion, such as during pregnancy, or increased vitamin D intake. Knowledge of the molecular features and physiological effects of individual urinary proteins is a prerequisite for determining their roles in disease.

The complex pathological process leading to the formation of urinary stones is best approached within the context provided by investigations of the more general process of biomimelarization. Living organisms utilize a diverse array of mechanisms for generating biomimelarized structures, including individual particles and single crystals, assembly of crystal composites, and fabrication of mineral-organic composite microstructures (48). Conversely, many organisms have the capacity for inhibiting potentially pathological crystallization. Thus biomimelarization serves two distinct functional roles.

The first role is in the assembly of the extracellular systems required by the organism (i.e., formation of calcium carbonate shells and carbonated hydroxyapatite teeth and bones) (17, 39, 40). Current concepts of the assembly of rigid structures suggest that calcifying organisms use protein templates and modulators to direct nucleation and growth. Alternatively, biomimelarization restricts the amount of potentially injurious mineralized products. In this second role, protein-crystal interactions may function in a protective fashion by either partially or fully inhibiting mineralization. Inhibition of ice formation by proteins in the blood of arctic fish provides an analogous example of such protection.

A critical and largely uncharted aspect of biomimelarization involves the mechanisms exerting control at a molecular level over crystallization at biological surfaces. Thus defining these
Molecular-Level View of Crystal Growth

To understand the mechanisms of inhibition of COM growth by citrate and OPN, a molecular-level view of crystal growth is required. In general, biomineralization is synonymous with biological control over crystal nucleation, growth kinetics, and morphology. Thus a key to a more fundamental understanding of the control of normal and pathological mineralization in biological systems is quantitative molecular-scale definition of the impact of modulators on the crystallization process. Current concepts of crystallization at a molecular level have been derived by analysis of classic crystal growth models within the perspective afforded by more recent interferometric, AFM, and molecular modeling investigations. These concepts have been recently reviewed in detail (16) and are briefly summarized in this section.

Fig. 1. Illustration of growth at crystal surface by attachment of molecules to step edges on either islands (A) or dislocation spiral (B). C and D: sequential atomic force microscopic (AFM) images showing portion of growth spiral with $L < L_c$ at $t = 0$ and $L > L_c$ at $t = 25$ s. Step edges with arrows are advancing. $L$ is length of a step edge, $L_c$ is critical length of a step edge, $a$ is distance between 2 adjacent atoms in crystal lattice, $v$ is velocity of step movement, $R$ is growth rate, and $h$ is step height. (Modified from Ref. 16.)
crystals (55). As this figure shows, COM crystals typically express three major families of crystallographically distinct faces, i.e., the \{−101\}, \{010\}, and \{120\}, although under certain conditions others are also possible (10, 13, 31, 43, 69). AFM images of the \{−101\} and \{010\} faces in Fig. 2, C and D, and of the \{120\} face in Ref. 31 show that COM crystals grow on step edges emerging from dislocations. These images also show that the steps form spiral arrangements of steps and terraces, called dislocation hillocks, whose shapes are face specific. On the \{−101\} faces, the steps form hillocks with triangular shapes, while on the \{010\} faces they form parallelograms. The basis for these spirals will be discussed after focusing on the processes occurring at the step edges.

The kinetics of the attachment/detachment process at step edges are determined by the density of kink sites and the energy barriers. In general, the barrier to desolvation, i.e., the breaking of bonds to solvent molecules, appears to be the dominant barrier to attachment of a solute molecule to the step. When there is detachment from a step, the primary barriers are the bonds to adjacent molecules in the crystal. The speed of step movement reflects these barriers. As the solute concentration (and resultant flux of solute to the step) is increased, step speed increases. The kinetic coefficient, (β), is the slope of this relationship. Determining the dependence of the kinetic coefficient on temperature allows calculation of the energy barriers that ultimately control the kinetics of growth (36). Moreover, when the value of the kinetic coefficient depends on solute concentration, the variation can be related to the rate at which new kink sites are created or existing ones are blocked. Thus measuring perturbations in the kinetic coefficient by modulators provides a quantitative assessment of atomistic mechanisms underlying alterations of growth kinetics (37, 64).

The notion of critical size is a crucial concept for understanding the probability of nucleation of islands, rate of crystal growth, and morphology of a growing crystal surface. A change in chemical energy per molecule, \(\Delta \mu\), occurs as molecules leave a solution and enter the solid state. Systems will always move toward a state of minimum free energy. Thus growth occurs when the change in chemical potential is negative, and dissolution occurs when it is positive. By convention, the change in chemical potential is expressed relative to that of the bulk crystal. Since growth occurs at the crystal surface where bonding is incomplete compared with the bulk, the drop in chemical potential resulting from transition to the solid state does not fall to the level within the crystal. Molecules attached to the surface have a relative excess of free energy called surface energy. Similarly, whenever a new step is created on a surface, for example during the birth of an island, there is an excess free energy known as step edge energy (γ). Free energy changes during step growth then reflect two terms: 1) a stabilizing term due to drop in chemical potential per attaching molecule and 2) a destabilizing term due to an increase in edge free energy. The first term is both negative and proportional to the number of molecules in the island, while the second term is positive and proportional to the length of the island perimeter. The difference goes to a maximum at an island radius referred to as the critical radius. Islands smaller than the critical radius decrease free energy by shrinking, while those larger than the critical radius decrease free energy by growing. Thus smaller islands dissolve and larger ones grow. The relationship between supersaturation and the critical radius of an island is expressed by the Gibbs-Thomson (G-T) relationship. Experimental determination of that relationship allows extraction of step edge energy, the most important thermodynamic factor controlling growth from solutions (16, 35, 64). Furthermore, measuring the equilibrium shape of an island or emerging step gives the orientational dependence of step edge free energy (51).

The concept of critical size also applies to step segments. A step segment advances as an adjacent new step edge is created. A segment of sufficient length advances because the molecules added along the step undergo a large enough drop in free energy to counteract the increase in step edge energy of the new step segment. As a step advances from a dislocation, the new side created along a step does not advance until a critical length is attained (Fig. 1, B–D). This side then advances and creates another new side. The end result is a polygonal spiral (called a growth hillock or dislocation hillock) with a terrace width that is determined by the critical length. At the relatively low supersaturations typically used in solution crystallization, the odds of achieving a large enough cluster of molecules to form an island exceeding the critical size are very small. Thus the impact of the G-T effect is that growth occurs exclusively at dislocation hillocks where steps always exist.

The G-T effect also plays an important role in control over crystal growth by modulators. Although there are numerous mechanisms by which modulators can affect growth, a model of inhibition originally developed by Cabrera and Vermilyea (C-V) (9) is particularly important for understanding the effects of citrate on COM. In the C-V model, each terrace acts as a
collection area for additives that adsorb to the crystal. As the steps advance across the terraces, they encounter these adsorbates. Adsorbates that bind strongly to steps in a way that disturbs the bonding of subsequent solute molecules create pinning sites (Fig. 3A). The steps bulge out between the pinning sites, and the resulting curvature reduces the local supersaturation through the Gibbs-Thomson effect. Such effects of citrate on morphology of steps on the (−101) face of COM are shown in Fig. 3B. At a high enough coverage, the radius of the step edge falls below the critical radius and the steps stop advancing even though the bulk supersaturation is still positive. The crystal is said to be in a “dead zone.” In addition, binding of adsorbates to kink sites can temporarily block solute attachment to those kinks without step pinning. This reduces the kinetic coefficient, even though the adsorbate spacing on the terraces is greater than the critical radius. The curves based on an updated C-V model of step pinning (68) that takes into account adsorption kinetics show excellent fit to the experimental data for citrate (Fig. 3C).

**AFM Investigation of Crystal Growth and Modulation**

In the context of the features and concepts illustrated above, the unique advantages of AFM as a tool for investigating modulation of nucleation and growth are more readily appreciated. AFM is the only tool that allows real-time investigation of solution crystal growth at length scales ranging from that of individual molecules to entire 100-µm crystal faces (35). At the longer length scales, growth mechanisms and morphology are easily assessed. At the length scale of individual atomic steps and terraces, step speed, step fluctuations, and molecular attachment/detachment rates can be probed. Molecular and atomic resolution is also achievable in many systems (15, 16, 35, 56). Additional unique advantages of AFM include 1) mineral surfaces can be imaged in aqueous solutions, whereas most surface analysis methods with nanometer scale resolution are limited to ultra-high vacuum conditions. 2) Solution conditions such as composition, temperature, pH, and flow rate can be precisely controlled, and solutions can be changed during the course of an experiment. This allows direct quantitative comparisons between the morphology and rates of nucleation and growth in pure solutions and those containing modifiers (15). Quantitative AFM studies have enabled incisive investigations of the effects of modulators on the morphology (14, 15, 21, 37, 51, 55, 56), kinetics (12, 21, 37), and energetics (51, 56) of crystal growth. In each of these studies, a new understanding of either the relationship between the additive and the crystal surface or the mechanism of growth modification was quantitatively established.

The relationships between molecular structure (13) and growth features on COM were clearly revealed by AFM (55). COM crystal shape and size depend on growth conditions, including ionic strength, pH, temperature, and local environment (10, 13, 31, 43, 69). Many crystal habits have been reported, but the dominant faces are usually some combination of the (−101), (010), (120), and (021) faces (Fig. 2, A and B). The geometry of growth hillocks on the (−101) and (010) faces is shown in Fig. 2, C and D, respectively. The (−101) face has only single elementary steps (height = 3.98 Å), while the (010) face also has double and quadruple steps (height ~16 Å). The genesis of the differences in steps on these faces has been discussed in detail (55, 56). Quadruple steps on COM appear to play an important role in growth modulation. They...
allow proteins that are too large to effectively interact with single steps to impact growth by effectively interacting with the taller quadruple steps (55).

Growth hillocks on the (-101) face exhibit a triangular morphology that differs from its equilibrium hexagonal habit (Fig. 2C). Growth in pure solution is very anisotropic, with [101]-directed steps moving ~10 times faster than [-1–20] and [-120]-directed steps; this gives rise to very different terrace widths (Fig. 2C).

Molecular Modeling of Crystal Modulation

AFM analysis of the changes in kinetic coefficient, step morphology, and step edge free energy due to growth modulators provides a means for determining the sites of interaction and mechanism of modification, but AFM provides little information about the underlying stereochemical relationships that drive modulation. Molecular modeling is an independent tool that serves this purpose. A single example that emphasizes interactions of inhibitors with crystal faces is briefly cited here to provide context. Molecular modeling and scanning electron microscopic (SEM) studies of the binding of citrate and phosphocitrate to COM crystals indicated that the (-101) face was the preferred site for binding of both citrate and phosphocitrate. Both additives had more complementary conformations and greater binding energies with this face than with the (010) face (73). However, more recently, the paramount role of step-modifier interactions was established (14, 51, 55, 56). For example, the modification of calcite by single enantiomers of aspartic acid results in a chiral habit that can only be explained on the basis of interactions at the step edges (51). Molecular modeling showed that interactions at the steps are far stronger than on the terraces. The source of strong interactions is the availability of coordination sites on both the step riser and the underlying terrace that accommodate the 3D structure of the amino acid. This principle proves crucial for understanding modulation of COM growth.

Role of AARPs

Although the chemistry of the proteins believed responsible for the control of mineral growth is complex and diverse, many are AARPs. For example, proteins from mollusk shells can contain >50% aspartate (23). A series of elegant in vitro studies have demonstrated tissue-specific patterns of modulation of crystal growth by AARPs extracted from mineralizing tissues (1, 7, 8). AARP solutions from mollusk shells interacted specifically with calcium dicarboxylic acid crystals to promote the growth of certain faces, while simultaneously slowing the overall rate of crystal growth (1). Protein mixtures from abalone shells switched the crystalline form of CaCO₃ slowing the overall rate of crystal growth (1). Protein mixtures promote the growth of certain faces, while simultaneously acted specifically with calcium dicarboxylic acid crystals to interaction of crystal growth by AARPs extracted from mineralizing tissues (1, 7, 8). AARP solutions from mollusk shells inter- modulation of crystal growth by AARPs extracted from mineralizing tissues (1, 7, 8). AARP solutions from mollusk shells inter- modulation of crystal growth by AARPs extracted from mineralizing tissues (1, 7, 8). AARP solutions from mollusk shells inter-

Inhibition of COM Crystallization by a Urinary AARP, OPN, and Citrate

OPN is a urinary AARP with a peptide sequence (60) identical to that of OPN from human bone (19). The OPN sequence contains several aspartic acid-rich domains (50); the domain from amino acid 70 to 105 of the human peptide chain contains 19 aspartic acids (53%) (27). In vitro studies have shown that OPN and polyaspartic acid share similarities in their interaction with CaOx. For example, the presence of either OPN or polyaspartic acid shifted the predominant crystalline form of CaOx from monohydrate (COM) to dihydrate (COD) (71, 72). This phase shift may be protective, since COD crystals show less binding than COM crystals to renal cells (72). As with other AARPs, the β-sheet structure of OPN appears to be an important feature. For example, the β-sheet structure of rat OPN isolated by immunoaffinity purification was significantly increased after exposure to calcium (28). Kinetic analysis showed that human urinary OPN inhibited COM growth by 50% at 16 ± 2 nM and inhibited the aggregation of COM crystals by 50% at 28 ± 4 nM (2), levels that are substantially exceeded by normal human urinary levels of OPN (2, 29, 44). OPN also inhibits nucleation (75) and attachment of COM crystals to renal epithelial cells (38) at comparable concentrations. Compositional and ultrastructural immunohistological analysis has shown that OPN is a major matrix component of COM stones (33, 41).

The posttranslational modifications of OPN provide a basis for regulation of mineralization. Effects on functional activities may result from local alterations in charge density or steric factors or to the conformational changes induced by the binding of divalent cations. Posttranslational modifications of OPN include phosphorylation (32, 54, 61), glycosylation (19, 54, 61), and sulfation (32). Phosphorylation of OPN primarily involves serine residues; many of the 42 serines in human OPN are in positions suitable for phosphorylation (62). A highly phosphorylated form of OPN is predominant in mineralized matrix and in bone-forming cultures (46). Phosphorylation of OPN appears directly pertinent to mineralization; inhibition of hydroxyapatite crystal growth by OPN was markedly reduced after dephosphorylation (30), and phosphorylation of OPN peptides markedly enhanced the inhibition of COM crystal growth (27). Polyaspartic acid also strongly inhibited COM growth, but like the phosphorylated OPN peptides showed only...
weak inhibition of COM crystal aggregation (27). It should be noted that the molecular features of full-length OPN protein molecules that are responsible for inhibition of crystal aggregation (2) have not yet been defined. Thus an understanding of the precise mechanisms by which OPN influences the several aspects of crystallization involved in urinary stone formation is only beginning to emerge. In any case, strong evidence for a pivotal role for urinary proteins such as OPN in the pathogenesis of urolithiasis has been provided by recent incisive gene ablation investigations. In these studies, hyperoxaluria was induced by giving ethylene glycol in the drinking water to mice with targeted disruptions of the gene encoding OPN. This caused renal CaOx deposits in the OPN knockout mouse model, but not in normal mice (70).

The concept that other urinary proteins that inhibit CaOx crystallization may also have important protective roles in urolithiasis is also quite plausible. Other urinary proteins identified as inhibitors of CaOx crystallization include nephrocalcin (47), prothrombin fragment 1 (63), bikunin (6), and calgranulin (53). Tamm-Horsfall protein (THP) was shown to be a potent inhibitor of COM crystal aggregation (25), while having much less effect on crystal growth (60, 76). As shown by OPN and THP knockout mice (45, 70), stone formation in humans could be fostered by quantitative deficiencies in protein synthesis. In addition, molecular abnormalities resulting from defects in primary sequence and/or posttranslational modifications could result in altered conformation and/or patterns of charge density crucial for crystal binding. Dysfunctional molecules could bind less well to crystals or could promote stone formation by other mechanisms such as acceleration of growth kinetics (21) or serving as nucleation sites.

An important role for urinary citrate in renal stone disease has been established by many clinical studies (52). A deficiency of urinary citrate caused by renal and gastrointestinal disorders is frequently observed in patients with CaOx stone disease. Furthermore, oral potassium citrate is effective as therapy for the prevention of recurrent stone disease (52). Citrate decreases the urinary saturation of calcium salts by forming complexes with calcium and thereby reducing the ionic calcium concentration. Citrate also has direct effects on the primary sequence and/or posttranslational modifications could result in altered conformation and/or patterns of charge density crucial for crystal binding. Dysfunctional molecules could bind less well to crystals or could promote stone formation by other mechanisms such as acceleration of growth kinetics (21) or serving as nucleation sites.

Molecular View of COM Modulation by Citrate and OPN

Addition of citrate markedly altered growth kinetics and morphology of steps on the (−101) face (55, 56). Figure 4 shows the temporal evolution of steps at a dislocation hillock during growth in the presence of citrate at a citrate-to-calcium ratio of 0.02. The [101] step speed eventually slowed by a factor of 25, and these [101] steps roughened dramatically. By comparison, roughening and slowing of other steps were significantly less. These other steps became rounded, but their speed was only halved. As a consequence, the resultant nearly isotropic step speeds changed the morphology of this face into a disk-shaped hillock (Fig. 4C). The measured dependence of step speed on citrate level and ionic calcium concentration exhibited both a dead zone and a reduction in kinetic coefficient (68). A comparison of the data with the C-V model of inhibition showed that it correctly predicted the measured dependencies (Fig. 3C). A comparison with measurements of inhibition in bulk solutions containing many COM crystals further showed that this molecular scale model can also be used in understanding the effect of citrate on macroscopic growth rates (66).

In contrast to what was observed on the (−101) face, citrate didn’t significantly change either morphology or step speed on the (010) face, suggesting that citrate interactions are much weaker on this face. Changes in the macroscopic appearance of COM induced by citrate (73) parallel those shown in Fig. 4C. Thus step-specific pinning on the (−101) face by citrate causes both inhibition of COM growth and modification of COM shape.

The stereochemical source of the highly step-specific interactions of citrate with COM was investigated by computer-based molecular modeling. Citrate is both nonplanar and contains three carboxylic groups. Citrate is small enough to be readily amenable to molecular modeling. Binding energies were calculated for citrate docking to the steps and faces of COM. The binding energies of citrate to each of the steps expressed during growth were considerably higher than to either the (−101) face (−65.4 kJ/mol) or the (010) face (−48.9 kJ/mol). The site that was most strongly affected by addition of citrate, the acute [101] step on the (−101) face, had the highest binding energy (−166.5 kJ/mol). The predicted geometry of citrate binding to this step is shown in Fig. 5A (56).

By comparison, binding energies for citrate to all steps on the (010) face were much less favorable. The predicted geom-

Fig. 4. Effect of citrate on morphology of the (−101) face during growth. Sequential AFM images obtained after introduction of citrate to growth solution showing progressive rounding of growth hillock and roughening of [101] step direction are shown. Time after introduction of citrate is $t = 0$ (A), $t = 10$ min (B), and $t = 33$ min (C). Solution conditions were $\alpha = 0.7$, citrate-to-calcium ratio = 0.02, and calcium-to-oxalate ratio = 1. Image size = 4.1 $\times$ 3.3 $\mu$m. Because the speed of the [101] step is more strongly affected than the [120] steps, its speed drops more dramatically and thus the terrace widths become nearly equal in the 2 directions. These changes and their dependence on citrate concentration have been previously discussed in detail (55, 56).
tery of citrate binding to the [−100] step is shown in Fig. 5B. Thus binding energies for these steps are also consistent with the very minor effects of citrate on the (010) face (55, 56).

The stereochemical relationships determined by modeling revealed the importance of both orientation of oxalate groups and configuration of calcium sites on the crystal surface for the binding of citrate molecules. The geometry of the acute [101] step on the (−101) face provides the most favorable steric configuration because it optimizes both factors. Specifically, the flat orientation of oxalate groups on the (−101) face avoids electrostatic repulsion of the citrate carboxylates by electro-negative ends of oxalate molecules. Spacing of calcium sites along the [101] step is also well matched to all three carboxylic acids of citrate, a molecule with a relatively rigid conformation (Fig. 5A). In contrast, on the (010) face, the extension of dicarboxylic acids of oxalate molecules beyond the (010) plane (Fig. 5B) causes electrostatic repulsion and thereby makes it difficult for citrate molecules to bind either to the steps or to the face. In addition, the 90° angle between the basal plane and the step riser results in a poor geometric match between the calcium ions and citrate carboxylates. In summary, the geometric relationships and molecular modeling calculations rationalize the AFM results. Strong citrate-step interactions lead to pinning of all steps on the (−101) face. However, differences in binding energies among steps on this face result in asymmetric reductions in step kinetics that cause the citrate-induced changes in COM morphology.

In striking contrast to the effects of citrate, OPN produced major morphological modifications and strong inhibition of step kinetics on the (010) face but had little effect on the step kinetics on the (−101) face (55). These changes were due to step-specific OPN interactions leading to step pinning. Figure 6 shows a sequence of AFM images collected on the (010) face during growth in a solution containing 5 nM OPN. After OPN was introduced, all steps became strongly pinned and lost lateral stability, with step speeds dropping by an order of magnitude. However, OPN did not alter either step speeds or step morphology on the (−101) plane. Although the interactions of OPN with steps on this face were apparently weak, discrete adsorbates appeared on the (−101) terraces at all OPN concentrations investigated (1–25 nM). Their dimensions did not change over time, but the number of adsorbates did increase over time (55). These findings indicate that OPN molecules interacted with the terraces strongly enough to form adsorbates but interacted weakly with the steps on the (−101) face and thus caused no changes in step kinetics or morphology.

As with citrate, the divergent effects of OPN on the (−101) and (010) faces must reflect differences in geometric relationships of functional domains with crystal steps and terraces. Dicarboxylic acid residues of the AARPs are known to be responsible for their strong interaction with crystal surfaces. Phosphorylation of OPN has also been linked to inhibition of mineral growth. However, protein binding to heterogeneous interfaces reflects both the charge density of functional groups and the spacing of these groups with relation to the local geometry of the mineral face and steps. Although OPN molecules are highly flexible in dilute aqueous solution (10 mM phosphate) (20), a fixed conformation may be induced by calcium ions (28) or by binding to the mineral surface, as suggested by the apparently inert nature of the OPN adsorbates on (−101) terraces. Among local characteristics that should play a role in OPN binding is the step height. On the (−101) face, step heights are only 6.0 Å. However, as described above, the periodic bunching of steps perpendicular to (010) face results in quadruple steps with a height of 16 Å (Fig. 2D). In contrast to the single-step heights seen on the (−101) face, the much greater height of the quadruple steps on the (010) face is expected to facilitate binding of a greater number of carboxylic acid and phosphate groups on the surface of the much larger OPN molecule to both the taller step risers and the basal plane. The resultant stronger interaction of OPN with steps on the (010) face would provide the basis for pinning of steps. On the (−101) face, the step risers are tall enough to satisfy the steric requirements for strongly binding citrate. However, they may not be tall enough for comparable binding of OPN. Thus, although binding to an individual site on a terrace may be weaker than at a step, relatively weak binding of OPN molecules to the planar terrace may dominate due to the high abundance of relatively smaller individual binding sites compared with the number of sites available at a single step.

Because OPN and citrate have their major effects on different COM crystal faces, they should have additive effects. Indeed, constant composition kinetics analysis showed that when levels of citrate and OPN needed to independently reduce the growth rate by 50% were both added to the solution, the
growth rate was reduced to \(-25\%\) (67). COM crystal morphology after exposure to this combination of OPN and citrate also reflected an additive effect by displaying rounded, poorly formed facets in both the (010) and (101) planes.

In summary, the combination of AFM and molecular modeling studies has defined both the physical and stereochemical factors responsible for COM inhibition by citrate and OPN. The results show that these two investigative tools, which have been used successfully to investigate growth in a wide range of crystal systems (15, 16), can reveal new insights about bimineralization. With respect to stone disease as well as other cases of pathological mineralization, these insights provide a clear basis for understanding control by small molecules and proteins. We predict that, as more powerful computers come online and protein structure determination becomes more routine, the combination of AFM and molecular modeling will facilitate the rational design of new therapies for pathological mineralization.

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