Crystal Surface Adhesion Explains the Pathological Activity of Calcium Oxalate Hydrates in Kidney Stone Formation

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Renal tubular fluid in the distal nephron of the kidney is supersaturated with calcium oxalate (CaOx), which crystallizes in the tubules as either calcium oxalate monohydrate (COM) or calcium oxalate dihydrate (COD). Kidney stones are aggregates, most commonly containing microcrystals of COM as the primary inorganic constituent. Stones also contain small amounts of embedded proteins, which are thought to play an adhesive role in these aggregates, and they often are found attached to the tip of renal papilla, presumably through adhesive contacts. Voided urine, however, often contains COD in the form of single micron-sized crystals. This suggests that COD formation protects against stone disease because of its reduced capacity to form stable aggregates and strong adhesion contacts to renal epithelial cells. Using atomic force microscopy configured with tips modified with biologically relevant functional groups, we have compared the adhesion strengths of the morphologically important faces of COM and COD. These measurements provide direct experimental evidence, at the near molecular level, for poorer adhesion at COD crystal faces, which explains the benign character of COD and has implications for resolving one of the mysteries of kidney stone formation.

Kidney stone disease, which occurs in >10% of the US population, causes substantial suffering and occasional renal failure, yet the disease mechanism is poorly understood. Calcium oxalate monohydrate (COM), the thermodynamically most stable form, is observed more frequently in clinical stones than calcium oxalate dihydrate (COD), at a ratio of >2:1 (1). Adverse physiologic effects are correlated with the presence of COM in the urinary tract, and COM retention within the renal tubules of rat kidney sections and in hyperoxaluric patients has been demonstrated. Macromolecules isolated from normal urine (i.e., healthy individuals) inhibit COM crystal growth in vitro, favoring formation of COD over COM, crystallization in normal urine typically affords COD, and COM adheres more than COD to renal epithelial cells in culture (2). Recent experiments using mice that were genetically altered to suppress osteopontin, a urinary protein thought to inhibit cell adhesion, demonstrated that only COM crystals were retained within the renal tubules (3). Conversely, asymptomatic individuals often have COD microcrystals in voided urine (4). The evidence strongly suggests that COD is a benign crystal form in the urinary tract, but the origins of its protective role are not understood.

Although crystallogenesis is essential to stone formation, calcium oxalate (CaOx) crystal growth rates are sluggish, to the extent that during typical urine transit times single crystals will not grow large enough to become lodged in the terminal collecting duct of the kidney (5,6). Consequently, crystal aggregation and attachment (of crystals or aggregates) to renal epithelial cells must represent critical processes in stone formation. Stones contain small amounts of embedded proteins, which are thought to serve as adhesive bridges between crystals in aggregates. Some in vitro studies have suggested that urinary proteins with substantial anionic functionalities serve as adhesives for COM and COD crystal aggregation and attachment to epithelial cells (7–10). These observations, coupled with the predominance of COM in stones and substantial amounts of COD microcrystals in voided urine, suggest that the difference in the pathologic behaviors of COM and COD is related to the adhesive character of their crystal faces, with COD less likely to form stable aggregates or strong adhesion contacts with epithelial cells. The adhesion strength of the crystal surfaces of these two forms, however, has never been compared.

To address this critical knowledge gap, we have compared the adhesion strength of various crystal faces of COM and COD using atomic force microscopy (AFM), wherein the force required to detach AFM tips modified with biologically relevant functional groups from the crystal surfaces is measured directly. This approach provides direct experimental evidence, at the near molecular level, for poorer adhesion at COD crystal faces, which explains the benign character of COD and has implications for resolving one of the mysteries of kidney stone formation.
Materials and Methods

The COM crystals used for adhesion force measurements were grown in vitro according to previously reported methods (11–13). The specific method was chosen to maximize the area of a particular crystal face so that AFM measurements were feasible. COD crystals with large (100) faces were grown in aqueous solutions containing 1.0 mM CaOx, 10 µg/ml poly(acrylic acid), 10 mM HEPES buffer, and 150 mM sodium chloride (Sigma Aldrich, St. Louis, MO). COD crystals with large (101) faces were grown in 1.0 mM CaOx and 5 µg/ml poly(acrylic acid), in the absence of buffer and salt. Characterization of the COM crystal surfaces and adhesion force measurements were executed with a Digital Instruments Nanoscope IIIa Multimode system (Digital Instruments, Santa Barbara, CA). All measurements were performed in aqueous solutions similar to those used for COD crystals grown by this method confirmed the existence of COD, and no unusual features were observed that would suggest inclusion of poly(acrylic acid) in the crystals, as has been suggested for proteins and amino acids in COM (14,15). Topographical and lattice images of the crystal surfaces were acquired with commercial Si3N4 cantilever tips in aqueous solutions containing 0.11 mM CaOx. Adhesion force measurements were performed in the same solution using gold-coated Si3N4 cantilevers with various thiol molecules. The spring constant of the AFM cantilevers, which is needed to convert the cantilever deflection to an adhesion force, was 0.074 ± 0.003 N/m, as measured by a previously reported method (16). The working solution, which was prepared immediately before the measurements and stored in a reservoir connected to the liquid cell via a Teflon tube, was refreshed at 10-min intervals to ensure uniform solution conditions. A typical adhesion force measurement with a given modified tip involved acquisition of 1000 individual force-distance curves recorded at 20 different locations on the crystal face. The force-distance curves were acquired at a rate of 2 Hz and with a loading force of 1 to 2 nN. The force-distance curves did not change perceptibly for acquisition rates in the range 0.2 to 2 Hz, nor did they change with loading forces up to 6 nN. The effects of these parameters outside these ranges were not examined. The adhesion forces were determined from the retraction portion of individual curves using customized software that automatically calculated the change in deflection upon detachment of the tip from the crystal surface. These were tabulated into histograms, and the mean values and SD were determined from the normal distribution curves. These were not appreciably different from the arithmetic mean values and SD. This procedure accounts for the likely possibility that the number of molecules adhering to the surface varies for individual force curves, while effectively averaging contributions from crystal surface defects and nonuniformities in the tip.

Results

Atomic force microscopy employs an ultrasmall tip, usually silicon or silicon nitride, located at the end of a silicon cantilever, which is brought into contact with a sample surface using piezoelectric actuators (17,18). As the tip is scanned across the sample, the surface topography can be visualized from the vertical motion of the tip, which is deduced from the position of a laser beam, reflected off the back of the cantilever, on a position-sensitive photodiode detector. This enables acquisition of two-dimensional images of crystal surfaces, which can reveal features such as terraces and steps that are related to the internal structure of the crystal. AFM also is capable of producing images of the truncated crystal lattice exposed at specific crystal faces. In another mode, the AFM can be used for direct and quantitative measurement of the force required to pull the AFM tip off a surface with which it is in contact. As the cantilever is retracted from the surface, it bends away from the surface while the tip remains in contact with the sample. The change in the deflection of the cantilever upon detachment of the AFM tip from the surface can be determined from the position-sensitive photodiode detector. If the force constant of the cantilever is known, the force of adhesion between the tip and the sample surface can be calculated. Both imaging and force measurements can be performed in a liquid medium, making this method ideal for examining biologically relevant processes. The force measurement capability has been used for quantitative measurement of adhesion forces between various molecular and biomolecular surfaces (19,20), but only recently has it been used to diagnose adhesion events at single crystal surfaces (21,22,23,24).

Through adjustment of crystal growth conditions, COM crystals for AFM adhesion force measurements were prepared with prominent (100), (121), or (010) faces, or COD crystals with prominent (100) or (101) faces (Figure 1; Miller indices, a three-digit notation used to define specific crystal planes, are indi-
cated). These faces were sufficiently large (5 to 10 microns) for positioning of the AFM tip on the surface. The Miller indices of these crystal faces and the adjoining faces were assigned with the aid of the crystal modeling program SHAPE (Shape software, Kingsport, TN). Topographic and lattice images of the COM faces, acquired with contact mode AFM in 0.11 mM CaOx solutions, indicated that their surfaces were crystalline, with structures identical to their corresponding bulk crystal planes (13). The COD (100) face revealed large terraces separated by $6.2 \pm 0.2 \text{ Å}$ steps, equal to $1/2a$ and corresponding to a single layer of calcium ($\text{Ca}^{2+}$) and oxalate ($\text{Ox}^{2-}$) ions, as expected from the crystal structure. The COD (101) face was less defined, exhibiting ridges rather than terraces with large areas. Although we have not yet been able to observe lattice images for either COD face, the well-formed (100) and (101) facets of COD crystals suggests that, like COM, the compositions and structures of its crystal surfaces can be deduced reliably from the bulk crystal structures (Figure 2) (25,26).

In the adhesion force measurements performed here, gold-coated AFM tips were modified with organosulfur molecules adorned with terminal carboxylate or amidinium groups, denoted Au:S(CH$_2$)$_{10}$COO$^-$ and Au:S(CH$_2$)$_{2}$NHC(NH$_2$)$^+$NH$_2$. These tips were chosen because they can be viewed as mimics of urinary protein segments (e.g., carboxylate mimics aspartate, glutamate; amidinium mimics arginine) that could promote adhesion between crystals in aggregates and attachment to epithelial cell membranes containing embedded proteins. Carboxylate groups on an AFM tip can also be viewed as mimics for Ox$^{2-}$ ions on a CaOx surface. In a given experiment, the AFM tip was brought into contact with a particular face of a CaOx crystal immersed in an aqueous solution of 0.11 mM CaOx. The adhesion force was measured from the change in the cantilever deflection when the AFM tip detached from the crystal surface during retraction. In this configuration, multiple molecules are contacting and separating from the crystal surface in each measurement. The mean adhesion force was determined from numerous individual force curves recorded at different locations on a particular crystal face.

In agreement with our previous observations (21), the mean adhesion forces decreased in the order COM (100) > COM (121) > COM (010) for both the Au:S(CH$_2$)$_{10}$COO$^-$ and Au:S(CH$_2$)$_2$NHC(NH$_2^+$)NH$_2$ tips. The adhesion forces measured with COD crystals, using the same tips under the same conditions, revealed that the adhesion strength of COD (100) was greater than that of COD (101), irrespective of the tip used (Figure 3, top and middle panels). Whereas the Au:S(CH$_2$)$_{10}$COO$^-$ tip binds more strongly to (COD) (100) than (COM) (121), the Au:S(CH$_2$)$_2$NHC(NH$_2^+$)NH$_2$ tip exhibits the opposite ordering. Overall, however, the adhesion forces scale with the Ca$^{2+}$ and Ox$^{2-}$ surface site concentrations deduced from the bulk crystal planes of COM and COD, as illustrated in the bottom panel of Figure 3 (the Ca$^{2+}$ and Ox$^{2-}$ in each crystal plane are nominally equivalent: COM (100) = 0.0542 sites/Å$^2$ > COD (100) = 0.0439 sites/Å$^2$ > COM (121) = 0.0429 sites/Å$^2$ > COM (010) = 0.0333 sites/Å$^2$ > COD (101) = 0.0225 sites/Å$^2$.

The comparable forces observed on each face for the oppositely charged Au:S(CH$_2$)$_{10}$COO$^-$ and Au:S(CH$_2$)$_2$NHC(NH$_2^+$)NH$_2$ tips—in identical solutions—supports equivalent surface concentrations of Ca$^{2+}$ and Ox$^{2-}$ ions and negligible contributions of coulombic interactions to the adhesion forces. This argues that the adhesion profiles for the Au:S(CH$_2$)$_{10}$COO$^-$ tip can be attributed to specific binding between the carboxylate group and surface Ca$^{2+}$ ions, whereas the adhesion profiles for the Au:S(CH$_2$)$_2$NHC(NH$_2^+$)NH$_2$ tip can be attributed to charge-assisted hydrogen bonding between the amidinium group and the carboxylate moiety of a surface oxalate (Figure 4). The N–H–H–N distance in the amidinium group (approximately 2.25 to 2.35 Å) and the O–O distance of the Ox$^{2-}$ carboxylate group (2.21 to 2.34 Å) are ideally matched for N–H–O hydrogen bonding as a heterodimer. The correlation of the adhesion force with Ox$^{2-}$ surface concentration suggests that the orientation of the Ox$^{2-}$ ions on the crystal planes does not substantially affect the adhesion to these tip molecules. The mean adhesion forces measured for bare gold tips or tips modified with a simple alkanethiol (Au:S(CH$_2$)$_n$CH$_3$) were approximately 1 nN, irrespective of the crystal face. Therefore, any adhesion force above this value can be attributed to specific binding. Notably, the adhesion force for COD (101) was approximately 1 nN for both tips, suggesting only minor contributions from specific binding.

Overall, these measurements reveal that the adhesion strength of the COM and COD faces, using biologically relevant...
functional groups as a probe, decreases in the order COM (100) > COD (100) = COM (121) > COM (010) > COD (101). This ranking establishes a crucial link between the pathologic behaviors of COM and COD and the adhesion strengths of their respective crystal surfaces, and supports the critical roles of aggregation and crystal adhesion to renal epithelial cells in stone formation. COM, the pathogenic form, exhibits large (100) faces when grown in urine-like media, and stones often contain stacks of COM plates emanating from a central nidus attached to renal cell surfaces. These stacks appear to form by crystal-to-crystal attachment of the large (100) faces. Thus, the most prominent COM face exhibits the largest adhesion strength, an ideal combination for creating robust COM aggregates and strong attachments to epithelial cell membranes that can persist under stresses experienced during flow in the renal tubules. Conversely, the protective form, COD, always exhibits large (101) faces when grown in urine-like media and in vivo, and the exposed area of COD (100) is minimal. Thus, the more adherant face, COD (100), is nearly inaccessible for adhesion contacts. Instead, the most prominent face, COD (101), displays the weakest adhesion strength. This condition would make COD aggregates and attachments to cell membranes less stable, thereby reducing the tendency to form stones, as reflected by the large amounts of individual COD microcrystals found in voided urine. Therefore, these adhesion measurements reveal, at a microscopic level, how COD formation can protect against stone disease. Furthermore, the monotonic dependence of the adhesion strength of the crystal faces with the surface concentration of Ca2+ (and Ox2− ions) for the COM and COD faces.

Figure 3. (top) Representative histograms for adhesion force measurements with the Au:S(CH2)10COO− and the COD (101) and (100) faces, performed in 0.11 mM CaOx solution at pH = 7. Each histogram corresponds to 1000 individual force measurements. (middle) The mean adhesion forces measured for the Au:S(CH2)10COO− and Au:S(CH2)10NHC(NH2)NH2 tips and the prominent COM and COD faces (see Figure 1). In this typical data set, the same tip was used for all the crystal faces. The asterisks (*) denote the most prominent crystal faces of COM and COD. Replicate measurements with different tips and crystals demonstrated that these adhesion force profiles were highly reproducible. The tips did not exhibit any appreciable change in adhesion forces during the measurements that would suggest deterioration of the tip. (bottom) Dependence of the mean adhesion force on the surface concentration of Ca2+ (and Ox2− ions) for the COM and COD faces.

Figure 4. Schematic representation of carboxylate and amidinium tips approaching binding sites on a CaOx crystal surface. For purposes of clarity only one molecule on each tip and one type of oxalate orientation are illustrated. The carboxylate probe docks to a surface Ca2+ ion and the amidinium tip binds to a surface Ox2− ion via charge-assisted hydrogen bonding.
ers (28), epithelial cell surfaces in culture (2,29), and nephrocalcin, a urinary glycoprotein (30), which suggests that the adhesion forces and binding affinities both depend on the number of accessible binding sites on the crystal surfaces. We anticipate that further studies of the kind described here, particularly when performed in the presence of urinary species thought to regulate stone formation, will further identify the key factors responsible for stone disease and may ultimately lead to preventative therapies.

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