Published in final edited form as:

Urology. 2011 September ; 78(3): 721.e13–721.e17. doi:10.1016/j.urology.2011.04.046.

ENZYMATIC DISSOLUTION OF CALCIUM AND STRUVITE CRYSTALS: IN VITRO EVALUATION OF BIOCHEMICAL REQUIREMENTS

Nabil K. Thalji, B.S.^a, Nigel G. Richards, Ph.D.^b, Ammon B. Peck, Ph.D.^c, and Benjamin K. Canales, M.D., M.P.H.^a

^a Department of Urology, Center for the Study of Lithiasis and Pathological Calcification University of Florida, Gainesville, FL

^b Department of Chemistry, Center for the Study of Lithiasis and Pathological Calcification University of Florida, Gainesville, FL

^c Department of Pathology, Immunology, and Laboratory Medicine, Center for the Study of Lithiasis and Pathological Calcification University of Florida, Gainesville, FL

Keywords

oxalate decarboxylase; nephrolithiasis; oxalate oxidase; purine nucleoside phosphorylase

INTRODUCTION

Dissolution of urinary tract stones using chemical agents and solutions, like Renacidin or Suby's solution, have been reported for over eighty years¹. Depending on stone composition, these agents are touted to work by lowering the pH of the solution around the stone and/or by exchanging minerals out of the stone (i.e. magnesium for calcium in apatite calculi) and into the surrounding solution. Unfortunately, the salts formed during pH changes and ion exchanges are quite insoluble, meaning constant irrigation is necessary for clinical effectiveness. This practice, known as chemolysis, has been discontinued largely due to advances in minimally invasive technology (shockwave lithotripsy, ureteroscopy, percutaneous stone extractions) and partially due to complications with the agents themselves (prolonged irrigation times, severe electrolyte disturbances, infection, death). Currently, the idea of chemically dissolving or augmenting calcium or phosphate-based stones is gaining considerable attention especially in patients who are poor surgical candidates or have recurrent stones.

Renal calculi are composed of a variety of organic and inorganic minerals which could be used as substrates by a variety of enzymes. Despite the poor solubility of stone material, we hypothesize that specific enzymes can convert small amounts of stone mineral into a more soluble product, enabling more mineral to dissolve. Eventually, based on the properties of catalytic chemistry, enzymes would drive the stone towards complete solubility (Figure 1), leading to dissolution.

In this work, we report an *in vitro* study of the biochemical requirements of enzyme-dependent stone dissolution to assess the viability of the approach. Since the composition of stones can vary greatly, we examined pure crystals of calcium oxalate monohydrate (COM), calcium phosphate dihydrate (brushite), and ammonium magnesium phosphate (struvite). For COM, we chose the enzymes oxalate decarboxylase (ODC) and oxalate oxidase (OxOx). Oxalate decarboxylase cleaves oxalate to form CO₂ and calcium formate (Figure

1a), which has much greater water solubility than calcium oxalate². Oxalate oxidase catalyzes the oxidation of oxalate to CO₂ and H₂O₂ (Figure 1b)². Since brushite and struvite stones both contain phosphate, the ability of the enzyme purine nucleoside phosphorylase (PNP) to dissolve both minerals was also investigated. PNP (Figure 1c) catalyzes phosphorolysis of purine nucleosides to yield ribose-1-phosphate and the free nitrogenous base³.

MATERIALS AND METHODS

Calcium oxalate crystal dissolution

COM crystals were made by precipitating a 1.0 M solution of NaOx with 1.0 M CaCl₂ and centrifuged to form pellets. Crystal structure was confirmed by light microscopy. COM pellets weighing 25 mg were combined with 10 mL of pH 4.2 buffered solutions (50 mM acetate, 50 mM NaCl), and 50 and 200 µg of ODC and 1 U of OxOx were added, respectively. ODC from *Bacillus subtilis* was overexpressed and purified via the methods of Reinhardt *et al*⁴. OxOx from *Hordeum vulgare* (barley) seedlings was obtained from Sigma-Aldrich (St. Louis, MO). In addition, for ODC studies, 10 mM *o*-phenylenediamine was added to enhance enzyme stability⁵. Simultaneous controls were also performed using the same setup, but without addition of enzyme. All samples and controls, performed in triple replicates, were incubated at 37°C. Dissolution was monitored daily using a calcium ion selective electrode (ISE) to measure calcium concentration⁶. The ISE was calibrated daily against six standard calcium solutions. For dissolution using ODC, calibration standards contained calcium derived from calcium formate dissolved in the same acetate buffer used for dissolution experiments. For dissolution using OxOx, calibration standards were prepared in nearly the same way, except that calcium chloride was used as the calcium source instead of formate.

Calcium oxalate product inhibition

To determine whether ODC dissolution of COM is limited by product inhibition, 10 U formate dehydrogenase (FDH; Sigma-Aldrich, St. Louis, MO) and 1 equivalent nicotinamide adenine dinucleotide (NAD⁺) were added to the dissolution reaction containing 200 µg ODC after the calcium concentration had failed to increase by more than 5% after five days. FDH catalyzes the NAD⁺-dependent oxidation of formate to form bicarbonate, and thus can consume formate produced by ODC to relieve product inhibition⁷. FDH and NAD⁺ were also added to the control mixture.

Phosphate mineral dissolution

The ability of PNP to dissolve brushite and struvite was examined by determining if the enzyme can utilize phosphate derived from these minerals as a substrate. Near saturation solutions (1.0 mM) of brushite and struvite powders (obtained from Sigma-Aldrich) were used as substrates for the EnzChek® Phosphate Assay Kit (Molecular Probes, Inc.; Eugene, OR) which utilizes PNP in its assay. Phosphorolysis was monitored spectrophotometrically by manufacturer protocol except 0.5 U of enzyme was used instead of 1.0 U. Rates were compared to the control initial rate of phosphorolysis using 1.0 mM potassium phosphate as the substrate. Data were collected using a Uvikon 810 (Kontron Instruments; Flowspec, Switzerland) spectrophotometer interfaced to a PC equipped with a data acquisition program, and all samples and controls were performed in triple replicates.

RESULTS

Dissolution of calcium oxalate by ODC and OxOx

Daily calcium ion concentration, measure to determine dissolution of COM crystals by ODC and OxOx, are plotted in Figure 2. Calcium ion concentrations were determined at each time point for samples containing 50 μ g ODC, 200 μ g ODC, and the corresponding controls. While controls appeared to show no significant change in calcium concentrations over a 15-day period, the samples containing ODC showed increases in calcium concentrations over a period of three to five days. After this initial period, the rate of increase slowed significantly. In the 50 μ g ODC sample, the calcium concentration reached a maximum of 1.57 mM after 15 days, corresponding to dissolution of 2.29 mg of COM. In the 200 μ g ODC sample, the maximum calcium concentration was 2.18 mM after 10 days, which corresponds to dissolution of 3.19 mg of COM. Controls, on the other hand, showed an average calcium concentration of 0.14 mM over the course of the experiments, or 0.20 mg.

Dissolution by OxOx was monitored in a similar fashion to dissolution by ODC. Calcium concentrations rose from 0.2 mM (day 2) to 0.52 (day 5) and peaked at maximum of 0.8 mM on day 15, corresponding to 1.17 mg dissolved COM (see supplemental data). For comparison, the average calcium concentration in the control sample was 0.07 mM, or 0.10 mg of dissolved mineral.

Examination of Product Inhibition using Formate Dehydrogenase

Since increases in calcium concentrations leveled off after three to five days when ODC was used to solubilize COM, it was suspected that product inhibition might be the cause. After five days without significant change in calcium concentrations in the 200 μ g ODC sample, formate dehydrogenase (FDH) and an equivalent amount of NAD^+ (with respect to the starting oxalate mineral) were added to consume the formate in solution, and the results are shown in Figure 3. Calcium concentrations increased rapidly on addition of the coupling enzyme and eventually reached a maximum concentration of 8.07 mM, 37 days after addition of the coupling enzyme. This concentration corresponds to a maximum dissolution of 11.79 mg COM mineral. In addition, the control sample (which was spiked with FDH and NAD^+ at the same time as the ODC-containing sample) had an average calcium concentration of 0.17 mM (0.25 mg) after addition of the coupling reagents.

Dissolution of brushite and struvite by PNP

The EnzChek® purine nucleoside phosphorylase (PNP) assay utilizes 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) as the nucleoside substrate to be cleaved by the phosphate. Initial MESG phosphorylase rates catalyzed by PNP were determined for 1.0 mM solutions of brushite, struvite, and phosphate standard. Each source was combined with buffer solution of 50 mM Tris-Cl, 1 mM MgCl_2 , 0.1 mM sodium azide, and 0.2 mM MESG substrate, and the reaction started by the addition of 0.5 U PNP. The velocities, presented in Table 1, are the average $\pm$ S.D. of three measurements. No statistically significant difference was demonstrated among the ranges ($\sim 80 - 90 \mu\text{M P}_i/\text{min}$) among all three groups ($p < 0.4$).

COMMENT

Enzymes have been shown in humans to be capable of dissolving mineral deposits. Most recently (2008), the enzyme uricase (PEGylated uric acid oxidase) was shown to be able to dissolve uric acid deposits in patients with chronic gout⁸. This systemic approach, now in Phase 3 clinical trials, works by reducing serum uric acid concentrations below saturating

levels and enabling precipitated mineral to dissolve. Using a similar approach, it seems logical that enzymes within the collecting system could also dissolve mineral precipitants.

ODC Dissolution and Formate Inhibition

In the current study, dissolution of COM pellets by ODC yielded favorable results, with significant changes in calcium concentration observed after the first day. Because the ISE was calibrated against calcium formate standards, it is reasonable to assume that these changes in calcium concentration are a direct representation of oxalate cleavage. In addition, the calcium concentrations found in the control solutions (between 0.07 and 0.17 mM) are consistent with the findings of Streit and colleagues⁶, who determined the solubility of various calcium oxalate hydrates in different solutions.

As might be predicted, the rates of dissolution could be controlled by the addition of more enzyme, consistent with fundamental principles of enzymology. Interestingly, after a few days, the rate of dissolution in both ODC samples dropped off drastically. Since one of the products of the ODC reaction is CO₂, the reaction can be assumed to be irreversible. Thus, the decrease in rate cannot be due to reaching the equilibrium position of the reaction. Furthermore, since the rate of dissolution in the sample containing 200 µg ODC began to level off before the rate of dissolution in the 50 µg sample, the decrease in rate is not likely caused by gradual inactivation of the ODC over time, since the sample containing more enzyme would be expected to retain enzymatic activity for at least as long, if not longer, than the 50 µg sample. Therefore, the most likely cause of the decrease in rate would be product inhibition by formate.

While product inhibition has not been previously reported for ODC, all enzymes do exhibit product inhibition to some extent⁹. Since formate strongly resembles the oxalate substrate, others groups have proposed that formate may inhibit ODC by chemical modeling studies⁵. High resolution ODC structural studies have also demonstrated binding sites for formate on manganese binding sites of ODC -- more evidence that formate has the ability to inhibit ODC enzymatic activity. Addition of the FDH coupling reagents resulted in nearly complete restoration of the maximum dissolution rate, suggesting that formate inhibition is the likely cause of the decreased rates observed. However, after 37 more days, the rate again leveled off. When 100 µg ODC were added to verify that this was not the result of ODC inactivation, the previous rate did not resume, suggesting that there may be another molecular mechanism at work in this system.

Product inhibition of FDH by NADH has been very well characterized, and, for the *Candida boidinii* enzyme, NADH is a competitive inhibitor with respect to the NAD⁺ substrate with a K_i of 30 µM¹⁰. Given the fact that the K_M of NAD⁺ for the FDH enzyme is 90 µM¹¹ and that 17.11 mM NAD⁺ was added initially, the velocity of the enzyme at the point where the dissolution curve levels off should be no greater than 27% of the initial velocity. Furthermore, since both NAD⁺ and NADH are nucleotides, they can be expected to decompose over time, particularly in aqueous solution at 37°C. The degradation products will most likely be other adenine nucleotides and nucleosides (ADP, AMP, and adenosine). ADP and AMP have been shown to inhibit FDH¹⁰, and this inhibition may contribute to the decreased rate as well. These two factors, therefore, probably account for the decrease in later stages of rate dissolution and suggest that, to achieve dissolution measurable by mass, the dissolution medium will need to be replaced periodically.

It is also notable that the catalytic rates observed for ODC reactions did not approach the maximal velocity of ODC derived from *Bacillus subtilis*. This is explained easily by noting that the K_M for oxalate reported in the literature for *B. subtilis* ODC is 15 mM⁵, while the solubility of COM in the buffer used (as determined from the control samples) was less than

0.2 mM. Under these conditions, the maximum obtainable velocity is less than 1.3% of the true $V_{\max}$ of the enzyme. Thus, *Bacillus subtilis* ODC may not be the ideal ODC source for COM dissolution. ODC produced by other organisms have been reported to have both lower K_M values (as low as 1.0 mM) and higher $V_{\max}$ values (twofold or more)⁵. More study is necessary to determine the ideal ODC enzyme for CaOx dissolution.

OxOx Dissolution

COM dissolution by OxOx yielded rather lackluster results, with maximum released calcium concentrations reaching less than half of the maximum concentration obtained in the uncoupled ODC study. This was rather surprising, since one product is CO_2 , and since the other product, hydrogen peroxide, has never been reported to exhibit product inhibition. However, it is possible that dissolved molecular oxygen, which is one of the substrates, is rapidly depleted, and that the rate of replenishment of more oxygen from the air is very low. If this is the case, one solution might be to add catalase, which would use the peroxide product to regenerate the substrate.

PNP Utilization of Mineral-Derived Phosphate

The initial velocity study using 1.0 mM solutions of brushite and struvite as phosphate sources indicated that phosphate derived from both minerals could be utilized by PNP in the phosphorolysis of MESG. The rates were slightly lower than the rate obtained when 1.0 mM potassium phosphate was the substrate, but the differences were negligible. This suggests that PNP may be useful in dissolution of stones containing these minerals. Unfortunately, a study similar to the one used for COM dissolution could not be performed with PNP because the equilibrium position¹² of the PNP reaction (using inosine as the nucleoside) would limit dissolution to the point that detection would be difficult and inaccurate. Although this experiment does not demonstrate dissolution of phosphate containing minerals directly, it suggests that effective dissolution of phosphate-containing minerals would be possible if 1) appropriate coupling reagents were used to drive equilibrium towards dissolution and/or 2) a different phosphate-utilizing enzyme were used.

From a feasibility standpoint, the use of these enzymes in an adjuvant setting for stone dissolution is still far away from clinical practice. We imagine this cocktail could be delivered intra-operatively, perhaps as the irrigation solution during ureteroscopy or in an adjuvant setting through a nephrostomy tube. Although most of the reagents and breakdown products should not cause local renal toxicity, clinical studies will be necessary before any comment can be made regarding safety or tolerability. Protocols utilizing acidic irrigation solutions like Renacidin (pH 3.85) have been extensively studied in the past and found to be safe if used properly¹³, so pH itself should not be a limitation. Further work in human oxalate and phosphate stone dissolution is ongoing by our group.

CONCLUSIONS

The current work provides the first example of a thorough *in vitro* evaluation of the biochemical requirements for crystal dissolution. For COM in a closed system, the only significant obstacle to ODC crystal dissolution is product inhibition which can be overcome by adding coupling reagents to deplete the inhibitory product. Despite low catalytic rates, ODC enzymatic activity is drastically increased by adding more enzyme. Dissolution of COM by OxOx was less successful, perhaps due to the absence of sufficient dissolved oxygen. This reaction may benefit from adding a coupling reagent that regenerates substrate. Finally, PNP can utilize brushite and struvite as a phosphate source, but the reversibility of the reaction limits the use of this approach. Even though coupling reagents could drive this

equilibrium towards dissolution, it may be more useful to utilize a different phosphate-consuming enzyme in future stone dissolution studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was funded through NIH grant R01 K061666 (NGR) and a Howard Hughes Medical Institute Undergraduate Fellowship Grant through the University of Florida (NKT).

References

1. Crowell AJ. Cystin nephrolithiasis; roentgenographic demonstration of disintegration by alkalization. *Surg Gynecol Obstet.* 1924; 38:87–88.
2. Svedruzic D, Jonsson S, Toyota CG, et al. The enzymes of oxalate metabolism: unexpected structures and mechanisms. *Arch Biochem Biophys.* 2005; 433:176–192. [PubMed: 15581576]
3. Webb MR. A continuous spectrophotometric assay for inorganic phosphate and for measuring phosphate release kinetics in biological systems. *Proc Natl Acad Sci U S A.* 1992; 89:4884–4887. [PubMed: 1534409]
4. Reinhardt LA, Svedruzic D, Chang CH, et al. Heavy atom isotope effects on the reaction catalyzed by the oxalate decarboxylase from *Bacillus subtilis*. *J Am Chem Soc.* 2003; 125:1244–1252. [PubMed: 12553826]
5. Tanner A, Bowater L, Fairhurst SA, et al. Oxalate decarboxylase requires manganese and dioxygen for activity. Overexpression and characterization of *Bacillus subtilis* YvrK and YoaN. *J Biol Chem.* 2001; 276:43627–43634. [PubMed: 11546787]
6. Streit J, Tran-Ho L-C, Konigsberger E. Solubility of Three Calcium Oxalate Hydrates in Sodium Chloride Solutions and Urine-Like Liquors. *Monatsh Chem.* 1998; 129:1225–1236.
7. Peacock D, Boulter D. Kinetic studies of formate dehydrogenase. *Biochem J.* 1970; 120:763–769. [PubMed: 4322039]
8. Sherman MR, Saifer MG, Perez-Ruiz F. PEG-uricase in the management of treatment-resistant gout and hyperuricemia. *Adv Drug Deliv Rev.* 2008; 60:59–68. [PubMed: 17826865]
9. Cleland WW. The kinetics of enzyme-catalyzed reactions with two or more substrates or products. III. Prediction of initial velocity and inhibition patterns by inspection. *Biochim Biophys Acta.* 1963; 67:188–196. [PubMed: 14021669]
10. Kato N, Sahm H, Wagner F. Steady-state kinetics of formaldehyde dehydrogenase and formate dehydrogenase from a methanol-utilizing yeast, *Candida boidinii*. *Biochim Biophys Acta.* 1979; 566:12–20. [PubMed: 215230]
11. Schute H, Flossdorf J, Sahm H, et al. Purification and properties of formaldehyde dehydrogenase and formate dehydrogenase from *Candida boidinii*. *Eur J Biochem.* 1976; 62:151–160. [PubMed: 1248477]
12. Porter DJ. Purine nucleoside phosphorylase. Kinetic mechanism of the enzyme from calf spleen. *J Biol Chem.* 1992; 267:7342–7351. [PubMed: 1559977]
13. Nemoy NJ, Staney TA. Surgical, bacteriological, and biochemical management of “infection stones”. *JAMA.* 1971; 215:1470–1476. [PubMed: 5107624]

Figure 1.

General proposed scheme for enzymatic-driven solubilization of renal calculi (top). Oxalate dissolution is proposed by enzymes oxalate decarboxylase (a) and oxalate oxidase (b). Inorganic phosphate (Pi) derived from mineral is proposed by purine nucleoside phosphorylase (c).

Figure 2.

Plot of calcium concentration over time as a measure of COM crystal dissolution by ODC. Dissolution curves utilizing 200 µg ODC (triangle), 50 µg ODC (circle), and superimposition of three control curves (dash) are shown. 2180 µM of calcium corresponds to 12.76% of the total COM or 3.19 mg dissolved. Lines are shown for ease of comprehension.

Figure 3.

Plot of dissolution of COM crystals by ODC (triangle) before and after addition of FDH and NAD^+ with simultaneous control (dash). Bold arrow indicates the point at which the coupling reagents were added. 8070 μM of calcium corresponds to 47% of the total COM or 11.79 mg dissolved. Lines are shown for ease of comprehension.

Table 1

Utilization of mineral-derived phosphate by PNP utilizing brushite and struvite compared to phosphate control. Reaction started with 0.5 U PNP. Velocities are the average $\pm$ S.D. of three measurements.

Phosphate Source	Initial Velocity ($\mu\text{M P}_i/\text{min}$)
Potassium Phosphate Standard	90.65 $\pm$ 1.38
Brushite	90.22 $\pm$ 1.17
Struvite	82.12 $\pm$ 3.57