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Identification of proteins extracted from calcium oxalate and calcium phosphate crystals induced in the urine of healthy and stone forming subjects

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Abstract The purpose of our study was to identify the proteins and investigate the differences, if any, between protein components of the matrices of calcium oxalate (CaOx) and calcium phosphate (CaP) crystals induced in vitro in whole human urine of healthy individuals and kidney stone patients. In addition, preliminary studies were performed to understand the effect of centrifugation and filtration of urine on its protein contents. Crystallization in urine was induced by addition of an oxalate or phosphate load. Crystals were collected, washed, and analyzed by scanning electron microscopy, X-ray diffraction, and energy dispersive X-ray microanalysis. Matrix proteins were obtained by demineralization with ethylene diamine tetraacetic acid (EDTA), analyzed by polyacrylamide gel electrophoresis, and identified by western blotting technique. No significant differences were detected between protein components of the matrices of CaOx and CaP crystals and between the crystal matrices obtained from the urine of normal and stone forming subjects. Albumin (AB), inter-α-inhibitor (IαI) related proteins, α-1 microglobulin (α-1 m), osteopontin (OPN), prothrombin (PT)-related proteins and Tamm-Horsfall protein (THP) were identified in matrices of both CaOx and CaP crystals induced in urine from both the normal subjects and stone formers. AB, PT-related proteins and OPN were the main constituents. The other proteins were present in smaller but detectable amounts. However, CaP crystal matrix, contained a large amount of THP. In addition CaP crystals contained significantly more proteins than CaOx crystals. Centrifugation and/or filtration of the urine resulted in reduction of many high molecular weight proteins including THP, AB and OPN in the urine.

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Introduction

Urinary stones are a mixture of crystalline and organic components. The latter represents 2%–5% of the dry weight of a stone [2] and is referred to as organic matrix. Electron microscopic investigations of human urinary stones [11–13, 16, 26] and calcium oxalate (CaOx) stones induced in rats [14, 15, 26] have demonstrated an intimate relationship between crystals and organic matrix. This association appears to develop early in the morphogenesis of the crystals, since organic matrix was seen occluded inside the "crystals" and the intercrystalline spaces, and the crystal nucleus appeared to contain more organic matrix than the surrounding areas [13, 15, 26].

Organic matrix of urinary stones may be derived from a variety of sources, including macromolecules of renal and serum origin, exfoliated epithelial cells, and cells originating in the blood [16]. What is the functional significance of various matrix components? Which of these components play a role in the formation of stones and which ones are incorporated secondarily? Just the presence of a compound in the stone matrix may not indicate that it fulfills an important role in stone formation. Clearly some matrix components play a significant role in stone formation while others do not. To separate these two classes of matrix components it was suggested [27, 28] that the matrix isolated from crystals experimentally induced in vitro in human urine should be investigated. Results showed a highly selective incorporation of urinary macromolecules. Tamm–Horsfall protein (THP) and albumin (AB), the two most common urinary macromolecules were absent from the CaOx crystal matrix. Using similar techniques, a 31-kDa protein was isolated from the CaOx crystal matrix and named crystal matrix protein [4]. This protein was shown to be a potent inhibitor of CaOx crystallization

[6] and was later found to be related to prothrombin and identified as prothrombin fragment-1 [31, 33].

Results of the above mentioned studies did not identify any other proteins such as osteopontin (uropontin) in the crystal matrices. Osteopontin (OPN) has several biological functions including involvement in biomineralization [5]. Amino acid sequence of OPN includes a high proportion of aspartic acid residues. It avidly binds calcium, is intimately associated with CaOx crystals in the urinary stones [26], and is a potent inhibitor of calcium oxalate crystallization [30, 34], and may be involved in crystal retention in the kidneys [24]. Based on the above observations it was apparent to us that osteopontin should be present in the matrices of CaOx and calcium phosphate (CaP) crystals. To verify this we investigated the organic matrix of CaOx crystals induced in normal human urine [1]. But unlike earlier studies where urine samples were centrifuged and filtered prior to induction of crystallization, we used whole urine, without centrifugation and filtration. We identified OPN and AB in matrices of CaOx crystals in addition to prothrombin (PT) fragments. Minor amounts of THP, α1-microglobulin (α1m) and light and heavy chains of inter-alpha inhibitor (IαI) were also detected.

CaOx is the major component of most urinary stones. As a result CaOx crystals have been the main subject of most such studies. CaP is the most common component of stones and may play a significant role in the formation of CaOx stones [18]. However, the matrix of CaP crystals has rarely been investigated [32]. In addition almost all studies so far have been limited to the matrices of crystals generated in the normal urine. But the urine of stone formers may have a different macromolecular composition than the normal. As a result, matrices of the crystals formed in stone formers' urine may have a composition different from those formed in the normal urine. An examination of matrix of crystals formed in stone formers urine may provide more information about the pathogenesis of stone formation. The current study was designed to analyze the matrices of CaP as well as CaOx crystals generated in the urine from normal as well as stone forming patients.

Materials and methods

Urine collection

Twenty-four hour urine specimens were obtained from a group of nine healthy persons, five males and four females, with no personal or family history of kidney stone disease and from nine calcific stone formers, six males and three females. No blood was noticed in any of the urine specimens and patients were radiologically stone free at the time. The urine samples were collected in plastic containers with sodium azide as preservative and were kept in the refrigerator during the collection period.

Crystal preparation

Each urine sample was divided into halves and each half was processed separately. Both halves were allowed to warm to 37°C in a shaking water bath (Fisher Scientific, Pa.). In one half, CaOx crystallization was induced by dropwise addition of 15 ml/l of 0.1 M sodium oxalate at native pH (about 5.9–6.5). CaP was induced in another half by addition of 15 ml/l of 0.1 M sodium phosphate after adjusting the pH to 7.2. The same amount of oxalate or phosphate was added at the end of 1 and 2 h. After 3 h of incubation, both urines were centrifuged at 10 000 rpm for 30 min at room temperature in a J2-21 centrifuge (Beckman Instruments Calif.). The crystals were harvested and washed three times with distilled water. Both CaOx and CaP were analyzed by scanning electron microscopy, energy dispersive X-ray microanalysis, and X-ray diffraction.

Matrix treatment

Crystals were demineralized by treatment with 5 ml of 0.25 M ethylene diamine tetraacetic acid (EDTA), pH 8 at 4°C for 3 days with continuous stirring. The extract was centrifuged at 10 000 rpm for 5 min and the supernatant was dialyzed against demineralized water for 24 h at 4°C using dialysis tubing with a 6–8 kDa cut-off (Spectrum Medical Industries Calif.).

Protein assay

Protein concentration in the matrix extract was estimated by the Lowry method [25]. Bovine serum albumin was used as standard.

Electrophoresis and western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 1-mm thick 10% ready mini-slab gel using Bio Rad Mini-Protean II apparatus (Bio Rad Laboratories, Calif.). Samples of 10 μg of protein and molecular weight standards were electrophoresed after they were heated for 5 min and subjected to β-mercaptoethanol reduction. Proteins were stained with Coomassie blue R-250 or transferred onto nitrocellulose membrane (Costar, Mass.) for immunological identification.

After electrophoresis, gels and nitrocellulose membranes were equilibrated in the transfer buffer (25 mmol/l TRIS, 192 mmol/l glycine, and 200 ml methanol). Proteins were transferred at 100 V for 1 h at 4°C using the same transfer buffer. Immunochemical staining was performed at room temperature by using different polyclonal antibodies. First of all, the nonspecific protein-binding sites were saturated by incubating the nitrocellulose membrane for 30 min in 1% serum albumin dissolved in TRIS buffer saline with Twin 20 (10 mmol/l Tris, 150 mmol/l NaCl, 0.5 ml Twin 20, pH 8). The membranes were incubated respectively with the primary antibody for 30 min. Polyclonal antibodies used at 1:1000 dilution are as follows: PT and AB (ICS Biomedical, Calif.), α1m (Binding Site, Calif.), IαI (Accurate Chemical Scientific Corporation, N.Y.). The latter antibody reacts with all three chains of IαI, heavy chains H1, H2, and light chain, the bikunin. PT antibody reacts with PT fragment-1, fragment 1 + 2 as well as PT. THP antibody was made in our laboratory by injecting rat THP into rabbit as antigen (Kel Farm Laboratory. Fla.) and OPN antibody was a gift from Dr E. M. Worcester (Department of Medicine, Medical College of Wisconsin, Milwaukee, USA). The membranes were then incubated with the second antibody marked with alkaline phosphates (Hyclone Laboratories, Utah) and used at a dilution of 1:5000. Finally, the membranes were allowed to develop for 5 min by using a substrate kit (Bio Rad).

Urine processing

Preliminary studies were performed to understand the consequences of centrifugation and filtration of urine on its protein profile. Whole urine was centrifuged at 10 000 g for 30 min or filtered through MSI acetate plus filters of 0.22 μm pore size before analysis by SDS-PAGE and western blotting.

Results

Analysis of crystals

Addition of sodium oxalate to the urine resulted in the formation of a mixture of monoclinic CaOx monohydrate and dipyramidal CaOx dihydrate crystals. Addition of sodium phosphate generated a poorly crystalline powder. X-ray microanalysis of this substance revealed the presence of calcium and phosphorus confirming it to be a calcium phosphate. There were no differences between the precipitates formed in urine of normals and stone patients.

Protein components of the organic crystal matrices

Figs. 1 and 2 show examples of matrix proteins extracted from CaOx crystals induced in normal and stone forming human urine respectively, analyzed by SDS-PAGE stained with Coomassie blue (lane 2) and the corresponding western blots (lanes 3–6) immunostained with different antibodies. On polyacrylamide gel two principal bands at molecular weights (MW) of about 32 and 67 kDa and four other bands were detected. On western blot, we detected two principal bands at MW of 32 and 48 kDa when PT antibody was used (lane 3). Another PT-positive band was seen at about 80 kDa, which was more pronounced in matrices of crystals produced in stone formers' urine than in non-stone formers urine. OPN (lane 4) and AB (lane 5) were detected in the same position at 67 kDa. THP was found in small amounts at 95 kDa (lane 6). IαI-related proteins and α1m were also detected (not shown).

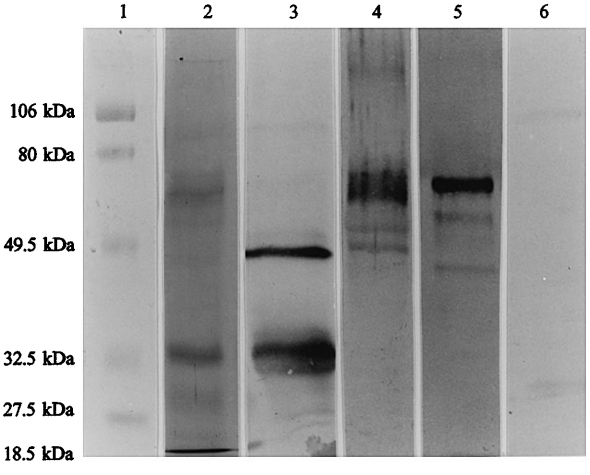

Fig. 1 Matrix proteins extracted from calcium oxalate (CaOx) crystals induced in normal urine. *Lane 1* Molecular weight (MW) markers, *lane 2* Coomassie blue stained SDS-PAGE with two main bands at MW 32 and 67 kDa. *Lanes 3–6* show western blots immunostained with various polyclonal antibodies: *lane 3* Prothrombin (PT) showing two bands at 32 and 48 kDa, *lane 4* Osteopontin (OPN) main band at 67 kDa, *lane 5* Albumin (AB) main band at 67 kDa, *lane 6* Tamm-Horsfall protein (THP) a very lightly stained band at about 97 kDa

Figs. 3 and 4 show examples of matrix proteins extracted from CaP crystals induced in normal and stone forming human urine respectively. On polyacrylamide gel several bands were seen (lane 2). The main bands appeared at 32, 67, and 95 kDa. On western blot, we detected two bands at a MW of 32 and 48 kDa when PT antibody was used (lane 3). Another PT-positive band

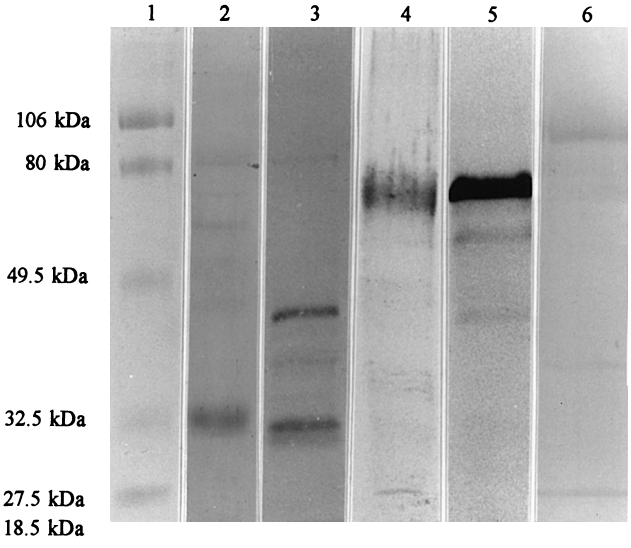

Fig. 2 Matrix proteins extracted from CaOx crystals induced in urine from stone patient. *Lane 1* MW markers, *lane 2* SDS-PAGE stained with Coomassie blue with main band at 32 kDa. Lanes 3–6 show western blots immunostained with various polyclonal antibodies: *lane 3* PT with two bands at MW of 32 and 48 kDa, *lane 4* OPN, *lane 5* AB, *lane 6* THP

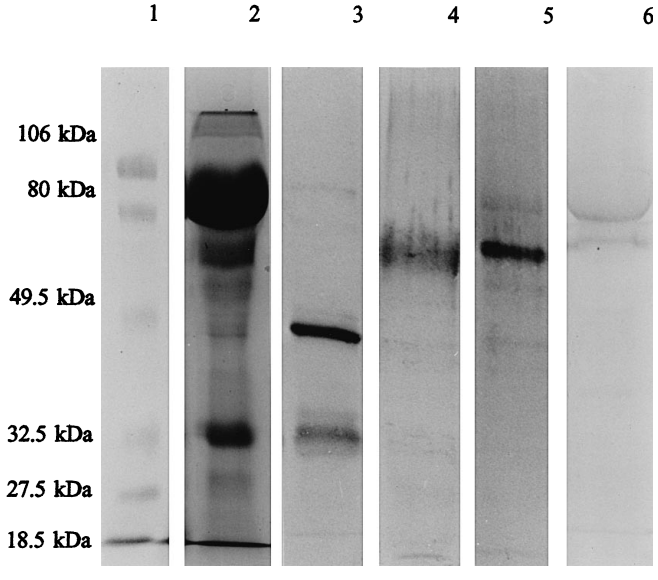

Fig. 3 Matrix proteins extracted from calcium phosphate (CaP) crystals induced in urine from normal controls. *Lane 1* MW markers, *lane 2* Coomassie blue stained SDS-PAGE showing main bands at 32, 67, and 95 kDa. Lanes 3–6 show western blots immunostained with various polyclonal antibodies: *lane 3* PT, two main bands at 32 and 48, *lane 4* OPN, *lane 5* AB, *lane 6* THP

was seen at about 80 kDa. Similar to CaOx crystals, this band was much more pronounced in matrices of crystals made in stone formers' urine. Principal bands of both OPN (lane 4) and AB (lane 5) were detected at 67 kDa. AB-positive bands were much more conspicuous in crystal matrices from stone formers urine than urine from non-stone forming subjects. Large amounts of THP were found at 95 kDa (Lane 6). IαI-related proteins and α1m were also detected (not shown).

Quantification of crystals and their matrix proteins

There were no significant differences between normal and stone patients in the amount of CaOx and CaP crystals generated in the urine and the amount of proteins extracted from the CaOx and CaP crystals (Table 1). Slightly more CaOx crystals were generated than CaP crystals in the same volume from both the normals and stone formers urine but the differences were not significant (*P*: 0.588 and 0.100 respectively). However, in terms of proteins extracted from crystals produced in 1 l of urine or per unit of crystal weight, CaOx crystals had significantly less proteins than CaP crystals (*P*: 0.001 and 0.001 respectively). For CaOx, the amount of matrix proteins ranged between 0.5%–0.7% of dry crystal weight in both normals and stone formers group. For CaP, the amount ranged between 2%–2.4% of crystal weight.

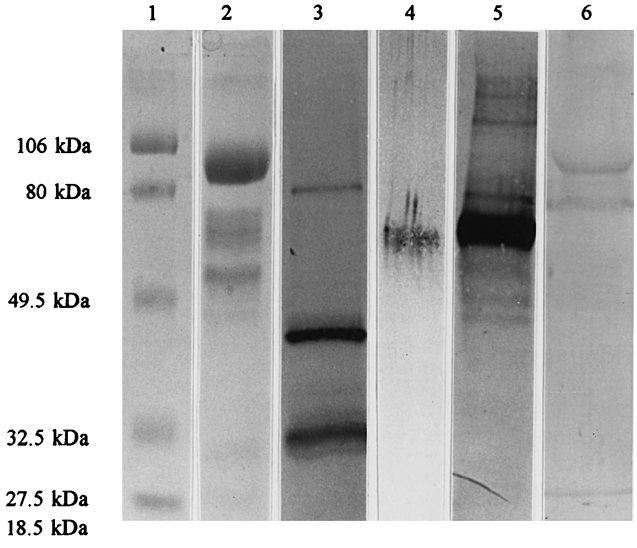

Fig. 4 Matrix proteins extracted from CaP crystals induced in urine from stone patients. *Lane 1* MW markers, *lane 2* Coomassie blue stained SDS-PAGE showing three main bands. Lanes 3–6 show western blots immunostained with various polyclonal antibodies: *lane 3* PT showing three bands 32, 48 and 80, *lane 4* OPN, *lane 5* AB, *lane 6* THP

Urine processing (results not shown)

SDS-PAGE analysis of the whole urine used for this study showed three major bands at 105, 82 and 49. Centrifugation resulted in a total loss of proteins of MW of 105 and an apparent reduction in the amount of proteins with MW of 82 and 49. Similar analysis of pellet from the centrifuged urine showed bands at 105, 82 and 49, indicating that centrifugation removed these proteins from the urine during processing. Filtration of urine similarly resulted in an apparent loss of proteins with MW of 105, 82 and 49. Western blot analysis indicated these proteins to be THP, OPN, and AB.

Discussion

It is well recognized that organic material is ubiquitous in both physiological and pathological calcified elements and influences the biomineralization processes. As far as urolithiasis is concerned, the role of organic matrix of kidney stones has long been investigated. However, its functional significance and its relationship to the kidney stone development remains uncertain. It has been proposed that a better understanding of the role of organic matrix in crystallization and urinary stone formation can be achieved by investigating in vitro induction of crystals in the urine. Studies of CaOx crystal formation by addition of oxalate load to normal human urine, demonstrated specific interactions between crystals and urinary proteins [4, 6, 27, 28]. There was selective incorporation of a 31-kDa protein in the crystal matrix while AB and THP were excluded. The protein was christened as the crystal matrix protein and later identified as prothrombin fragment-1. Results of other

Table 1 Amount of crystals produced in the urine of healthy normal controls and stone forming patients and the amount of proteins extracted from crystal matrix (±SEM)

Group	Crystal (mg/l)	Proteins (mg/total crystal per liter)	Proteins (μg/mg crystals)
Calcium oxalate crystals			
Normal	311.38 ± 38.73	1.6 ± 0.23	5.48 ± 0.66
Patients	339.32 ± 49.72	2.06 ± 0.3	6.55 ± 0.73
P	0.66	0.24	0.29
Calcium phosphate crystals			
Normal	245.29 ± 112.94	2.89 ± 0.55	21.13 ± 3.81
Patients	221.17 ± 45.81	4.13 ± 0.48	23.85 ± 4.29
P	0.85	0.11	0.64

studies in which CaOx crystallization was induced by mixing urine from healthy individuals with solution containing IM $CaCl_2$ and 0.1 M sodium oxalate [21], demonstrated that both urinary glycoproteins and glycosaminoglycans become associated with the CaOx crystals. These macromolecules were called crystal surface binding substances (CSBS). We have determined that urinary phospholipids also become associated with the CaOx crystals induced in vitro in human urine [17]. Ultrastructural studies have illustrated that individual crystals of urinary stones contain their own organic matrix which is present both on crystal surfaces as well as inside the crystals [11–16] and that the organic matrix of urinary stones comprises of crystal matrix and organic material present between the crystals. Thus crystal matrix is a sum total of all organic substances present in association with the crystals and may contain glycoproteins, glycosaminoglycans and phospholipids.

So far most studies have been limited to investigation of matrices of crystals produced in normal urine. Proteins in the urine of stone patients may be different from normal urinary proteins and may behave differently when interacting with the crystals. Stone formers may excrete different amounts of certain proteins. Moreover, even though CaOx stones predominate, CaP crystals and their associated macromolecules may play a critical role in CaOx urolithiasis by being involved in heterogeneous nucleation of CaOx [18]. The current study was undertaken to investigate the proteins in matrices of both CaOx and CaP crystals generated in the urine of both healthy persons and stone forming subjects. Our results showed that the amount of CaOx and CaP crystals produced in 1 l of urine and their organic matrix contents do not differ significantly between normals and stone patients. Also, in comparison between CaOx and CaP, the difference in the amount of crystals formed is not significant. However, in terms of protein matrix, CaOx crystals contained less proteins than CaP crystals. This may be due to the fact that CaP crystals are smaller and offer more surface area for protein binding than CaOx crystals.

No significant differences were found in the crystal matrix protein patterns between stone formers and non-stone formers challenging the hypothesis that urine of stone patients has a different protein profile than normal urine. However, it is still possible that there are differences between normal and stone formers' urine but not in the proteins that were investigated in this study. Moreover, there may be differences in the amounts of specific proteins excreted in the urine. As we indicated, SDS-PAGE analysis showed that crystal matrix from stone formers' urine produced a more pronounced band for prothrombin than crystal matrix from normal urine.

The matrix of CaOx and CaP crystals induced in the urine of normal subjects as well as stone patients, contained PT-related proteins comprising fragment-1 at 32 kDa, fragment 1 + 2 at 45 kDa, and in some samples, PT at about 80 kDa. Moreover, we also detected AB and OPN, both at 67 kDa. Small amounts of THP, α1-m, and IαI-related proteins including bikunin and H1 and H2 chains were also detected. This confirms our earlier finding [1] that the incorporation of urinary macromolecules into CaOx crystals is not limited to a single protein. This may be due to our use of whole urine without prior centrifugation and filtration in contrast to other studies where the urine specimens were either centrifuged or filtered or both [4, 27, 28, 31, 32]. Our preliminary results concerning the consequences of centrifugation and filtration on urinary protein profile described here show that these two processes remove many proteins from the urine. Obviously a filtered and centrifuged urine is not a whole urine. It is also important to point out that only six antibodies were used in our study which means that some other proteins may also be present in crystal matrix [7] but were not detected.

The principal proteins found in matrix of CaOx crystals formed in normal urine were PT-related proteins, followed by AB and OPN supporting the earlier contention that PT fragment-1 is the principal protein of crystal matrix extracted from CaOx crystals formed in normal urine [5, 31, 32]. However, it is not the only protein in crystal matrix and thus crystal matrix protein should not be considered synonymous with PT fragment-1. Both OPN and AB are also constituents of the crystal matrices. Ultrastructurally, OPN is a dominant component of the matrix of CaOx crystals in urinary stones [26]. The current study showed that albumin is more pronounced in matrix of crystals induced in stone formers' urine. This is in accordance with the recent results reporting AB to be a major component of the matrix of a variety of stones [7]. Furthermore, in a recent paper [32], the analysis of organic matrix of kidney stones by SDS-PAGE showed an intense band at 67 kDa corresponding to AB. Both CaP and CaOx crystals are known to adsorb AB [23, 27, 35].

THP appeared to be a major component of the organic matrix of CaP crystals. AB, PT-related proteins and OPN were the other important constituents. The matrix of CaP crystals induced in pooled normal human urine was recently shown to contain PT fragment-1 and a small amount of AB [32]. But the urine in which crystals were induced was first centrifuged at 10 000 g for 20 min and then filtered through a 0.2-μm filter, processing that would remove almost all high MW proteins.

Crystal retention within the urinary tubules is a determining factor for the formation of kidney stones. According to calculations based on renal tubular dimensions and the concentration of oxalate in the urine, single crystals of CaOx cannot grow large enough, during the normal transit time of urine, to be retained in renal tubules [22]. Presence of large amounts of AB and other proteins in the crystals produced in the urine of stone formers may increase crystal size and the volume of crystal aggregates, thereby increasing their chances of blocking renal tubules and being retained in the kidneys.

Several urinary macromolecules inhibit the adhesion of CaOx monohydrate crystals to renal epithelial cells, but AB does not [24]. Therefore, large amounts of AB found in association with crystals of stone formers may

increase the likelihood of crystal adherence to the renal cells and initiate stone formation. In addition, AB adsorbed to the crystal surfaces may also promote subsequent crystallization. In vitro studies have shown that many immobilized macromolecules can act as heterogeneous nucleators of crystals [3]. Hydroxyapatite crystals with a coating of AB were a better nucleator of CaOx monohydrate from a metastable solution than the pure inorganic hydroxyapatite [8].

A variety of proteins are present in the human urine and the matrix of human urinary stones [2, 7], but the amounts of various proteins in the stone matrix do not reflect their urinary concentrations. Some proteins are present in disproportionately higher quantities in the stone matrix while others are in much lower quantities than their concentration in the urine. THP is abundant in the urine but is scant in CaOx stone matrix. On the other hand urinary PT is present in small quantities in the urine but concentrates in CaOx and CaP crystals and has been detected in matrices of calcific stones [32]. There is a variation in the amount of organic matrix present in different types of stones as well as the kind of macromolecules included. On a dry weight/weight basis pure CaOx stones contain less organic matrix than stones with CaP [29]. More OPN is present in CaOx monohydrate stones than in CaP and CaOx dihydrate stones [10]. Results of our study showing differences in amounts of various proteins incorporated in CaOx and CaP crystals are consistent with these findings. Moreover, similar to the kidney stone matrix, our results also showed higher protein matrix associated with CaP crystals than with CaOx crystals. In addition, the six proteins we detected in matrices of CaOx and CaP crystals have all been found in matrices of calcific stones.

The data presented here clearly demonstrate that many macromolecules including PT-related proteins (fragment-1, fragment 1 + 2 and PT), OPN, AB, THP, α1 m, and IαI-related proteins (heavy and light chains) become a part of the crystal matrices. Interestingly almost all of these have been shown to influence CaOx crystallization [20]. Which of the matrix components play significant role in stone formation and which are included as mere bystanders? Obviously the matrix macromolecules that are found associated with crystals, and in functional assays, alter crystal habits, inhibit crystal growth and aggregation, and promote crystal nucleation and retention, are of vital importance. Urinary macromolecules such as membrane phospholipids, which can promote nucleation of calcific crystals [17], PT fragment-1, OPN, bikunin, which can inhibit crystal growth, and THP [9], which modulates crystal aggregation; all play significant roles. Thus it can be concluded that crystal matrix consists of a heterogeneous group of macromolecules many of which participate in stone formation by regulating different steps involved in the process [19]. It must be realized that functional and structural redundancy is a common phenomenon and the presence of only a single macromolecule capable of regulating crystallization in the urine will be an aberration.

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