Inclusion of Proteins into Calcium Oxalate Crystals Precipitated from Human Urine: a Highly Selective Phenomenon

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The abundance of protein in the matrix of calcium oxalate uroliths has fueled speculation regarding its role in stone genesis. In this study, we wanted to characterize the composition of the proteins associated with early stages of calcium oxalate crystallization in urine. Calcium oxalate crystallization was induced in urine from healthy men and women by the addition of an oxalate load. The crystals were harvested and demineralized, and the proteins remaining were separated and characterized by polyacrylamide gel electrophoresis and Western blotting. Most urinary proteins were not detected in the crystals or were present in only small quantities. The most abundant urinary macromolecule, Tamm–Horsfall glycoprotein, was notably absent from the crystal extracts. The predominant protein associated with the crystals, a previously unknown urinary constituent that we call crystal matrix protein (CMP; molecular mass, 30,000 Da), was more prevalent in the crystals derived from female urine. We conclude that most urinary proteins play no direct role in calcium oxalate crystal formation. However, the protein CMP exhibits a remarkable affinity for calcium oxalate crystals and may be important in stone pathogenesis.

Approximately 2–6% of the dry weight of renal stones composed principally of calcium oxalate is nondialyzable organic material (1–3). By analogy with other biomineralization systems, this material is referred to as the organic matrix. This matrix resides throughout the stone in a seemingly ordered pattern of fibrous concentric laminations and interdigitating radial striations that surround crystalline material (4, 5). About 64% of the matrix has been reported to be protein (6). Although characterization of this protein may help to clarify the role of matrix in stone genesis, only ~25% of lyophilized matrix is readily soluble (7), which has seriously impeded identification of the individual proteins in the matrix. Elucidation of the composition of the remaining 75% remains as elusive today as it was 20–30 years ago.

A further factor, namely, the source of the matrix, has also complicated the issue. Matrix inclusion can be conceived as two separate events, leading potentially to two distinct types of matrix. The first of these comprises nucleation and early growth of calcium oxalate crystals within the renal tubules. Such crystals undoubtedly contain a matrix of essentially normal urinary organic components (8, 9), in proportion to their binding affinity for the crystal surface (10). However, once these crystals have attained a microscopic size and have lodged in a kidney tubule, they have the capacity to induce the production of a second type of matrix by abrading the urothelial lining. This second event in matrix formation, trauma, will inevitably alter the macromolecular composition of the urine bathing the crystals and will result, again depending on binding affinities, in the deposition of a second type of matrix component chemically distinct from that occluded into the crystal during nucleation and early crystal growth. That such matrix inclusion does occur as a result of cellular injury is attested to by the identification in stone matrix of (a) serum proteins too large for glomerular filtration (7, 11), (b) lipids—a very nominal component of urine (12, 13), and (c) erythrocyte and mitochondrial ghosts and bacteria (14). In keeping with this concept, note that hematuria is virtually an invariable accompaniment to urolithiasis.

Although both the formation of crystal particles and their subsequent retention within the renal collecting system may be regarded as essential to stone pathogenesis, one can argue that the critical step is the first of these because the second is dependent on it. Study of the matrix material included specifically during the critical step of crystal formation has obvious advantages, primarily avoiding the presence of proteins included secondarily from trauma. This approach was recently adopted by Morse and Resnick (15), who used twodimensional gel electrophoresis to analyze the protein component of calcium oxalate crystals newly precipitated from human urine. The results of their study indicated that the occlusion of normal urinary proteins into the crystal is a selective phenomenon, because the amounts of individual proteins present in the crystals did not mirror their relative concentrations in the original urine. However, before inducing crystallization, Morse and Resnick centrifuged and filtered their urine specimens, which would have caused an almost complete loss of Tamm–Horsfall glycoprotein (THG) (16, 17), the most abundant protein in human urine, and some reduction in the concentration of albumin and other urinary proteins (18). Considerable disagreement has surrounded the role of THG in calcium stone formation: it has been reported to both promote (19) and

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2 Nonstandard abbreviations: THG, Tamm–Horsfall glycoprotein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBST, Tris-buffered saline with Tween 20 (see text); and CMP, crystal matrix protein.
inhibit (20, 21) calcium oxalate crystallization in vitro; evidence for its presence in stone matrix has also been contradictory (3, 22–24). Thus the first aim of this study was to compare the protein content of calcium oxalate crystals freshly precipitated from whole human urine with that of crystals derived from the same urine from which the THG had been removed by centrifugation (17) and filtration (16). Moreover, because women are well known to have a lower incidence of stone disease than men, because female urine specimens reportedly inhibit calcium oxalate crystal aggregation more potently than does male urine (25), and because proteins are known to inhibit calcium oxalate crystallization (26–28), our second objective was to compare the protein contents of normal male and female urines and that of the calcium oxalate crystals precipitated from these urines.

Materials and Methods

Materials

Chemicals. All reagents used were of analytical purity. Unless stated otherwise, the biochemicals were supplied by BDH Chemicals Australia (Kilsyth, Victoria, Australia). Other reagents were obtained from the following sources: EDTA, silver nitrate, trichloroacetic acid, and methanol (Univar-grade; Ajax Chemicals, Auburn, NSW, Australia); Tris, Tween 20, 2-(N-morpholino)ethanesulfonic acid, and electrophoretic-grade acrylamide and bis-acrylamide (Sigma Chemical Co., St. Louis, MO); Coomassie Blue R250 (Bio-Rad Labs., Richmond, CA 94804); bromphenol blue (Merck, Darmstadt, F.R.G.). All solutions were prepared with the highest quality water from a “Hi Pure” water purification system fitted with a 0.2-μm pore-size filter (Permutit Australia, Brookvale, NSW, Australia). High- and low-Mr electrophoretic standards were obtained from Pharmacia (Uppsala, Sweden).

Polyclonal antibodies. Unless otherwise stated, all immunological studies were performed with polyclonal IgG fractions of the corresponding antisera. Alkaline phosphatase (EC 3.1.3.1.)-conjugated sheep anti-rabbit, donkey anti-sheep/goat, and sheep anti-mouse fractions were obtained from Silenus Labs. (Hawthorn, Victoria, Australia), as were sheep anti-human α, acid glycoprotein and anti-human albumin. Other immunoglobulins and suppliers were: sheep anti-human THG (ICN Immuno Biologicals, Lisle, IL 60532); rabbit anti-human IgG, reacting mainly with γ, χ, and λ chains (Dakopatts, Dako Immunoglobulins A/S, Copenhagen, Denmark); rabbit anti-human IgG and complement component C3, reacting with all light chain structures (Dade Diagnostics, Aguada, PR); sheep anti-human apolipoprotein A-I (Boehringer Mannheim GmbH, Mannheim, F.R.G.); monoclonal mouse anti-human β2-microglobulin was a gift from Ms. R. Comachio and Dr. A. Hohmann (Department of Immunology, Flinders Medical Centre, South Australia).

Procedures

Collection of urines. During the same 24-h period, urine samples were collected without preservative from four healthy men (ages 25 to 43 years) and five healthy women (ages 18 to 41 years). The urine was refrigerated for the duration of the collection. Each specimen was checked for microscopic hematuria by use of Multistix test strips (Miles Laboratories, Mulgrave, Victoria, Australia); none showed any evidence of blood.

The urines were sieved through a 20-μm pore-size nylon mesh (Nyta1 HD20, Swiss Screens, Sydney, Australia) and pooled according to sex. Each pooled urine was divided into three equal portions. One portion was stored at 4 °C (sieved urine); the other two were subjected to further treatment.

Urine treatment. Further urine treatments were performed at room temperature. The urine specimens were centrifuged at 10 000 × g for 30 min in a J2-21M/E centrifuge (Beckman Instruments, Palo Alto, CA). Microscopic examination of the sediment revealed only cellular debris and precipitated protein. After filtration through 0.22-μm pore-size filters (GSPWP04700; Millipore Corp., Bedford, MA), one portion (centrifuged and filtered urine) was stored at 4 °C for further study and the other (ultrafiltered urine) was ultrafiltered through a hollow-fiber bundle (H1P10-20; Amicon Corp., Danvers, MA), with a nominal molecular mass cutoff of 10 kDa. The urines were analyzed immediately after treatment.

Estimation of urinary metastable limit and crystal preparation. The experimental system used for measuring a urine’s metastable limit with respect to calcium oxalate is described in detail elsewhere (29). Briefly, the metastable limit, the minimum amount of oxalate required to produce crystals detectable by the Coulter Counter (Coulter Electronics Ltd., Harpenden, Herts., U.K.), is determined by titrating aliquots of urine with sodium oxalate, followed by incubation at 37 °C for 30 min in a shaking water bath. The number of crystals >2 μm in each sample is then determined by using a Coulter Counter Model TALL fitted with a Population Count Accessory.

Once the metastable limit had been determined, we allowed 1 L of each pooled urine to warm to 37 °C in a shaking water bath and induced crystallization by drop-wise addition of sodium oxalate to a final concentration 30 μmol/L in excess of the metastable limit. The same amount of sodium oxalate was added at the end of 1 h and again at the end of 2 h to increase the yield of crystals. Incubation was continued for a total of 3 h.

Crystal isolation. Crystal isolation was performed at room temperature. Because normal urine cannot be filtered, given the presence of highly aggregated THG (16–18), we isolated the crystals generated in the sieved urines by centrifuging at 10 000 × g for 20 min in a Beckman J2-21M/E centrifuge. On the other hand, because the centrifuged and filtered and the ultrafiltered urines contain virtually no THG, we isolated by 0.22-μm Millipore filtration the crystals generated in these samples.

The isolated crystals were associated with small amounts of residual urine. To ensure that any extrane-
ous proteins present in the urine did not contaminate those in the crystal structure, we washed the crystals with dilute NaOH. Calcium phosphate precipitates from urine at slightly alkaline pH values, so we suspended the crystals in 50 mL of 10 mmol/L 2-(N-morpholino)ethanesulfonic acid buffer (pH 5.5) and vigorously vortex-mixed them before washing. We then harvested the crystals by filtration, washed them off the filter surface with 0.1 mol/L NaOH, and vortex-mixed them with 30 mL of the same solution. These washings were repeated for eight cycles; in the final two cycles we substituted distilled water for the NaOH.

Demineralization. To demineralize the crystals, we stirred 30 mg of each crystal preparation in 7 mL of 0.25 mol/L EDTA solution (pH 8.0) for three days at 4 °C.

Electrodialysis. The crystal extracts, or 8-mL aliquots of each urine, were placed in dialysis tubing having a cutoff of 6–8 kDa (Spectrapor Membrane Tubing; Spectrum Medical Industries, Inc., Los Angeles, CA 90054). Using a flat-bed electrophoresis tank with a 2-L buffer capacity, we subjected the specimens to electrodialysis at 4 °C against a solution of 25 mmol of Tris and 192 mmol of glycine per liter (pH 8.3) for 6 h at 80 V. The Tris/glycine buffer was replaced with water, and electrodialysis was then continued at 100 V, with three changes of water, for another 24 h. The samples were lyophilized to yield a colorless flocculant material that we processed as described below.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Material derived from the urine samples was dissolved in 250 μL of buffer containing 20 mmol of Tris, 2 mmol of EDTA, 20 g of SDS, and 100 mL of 2-mercaptoethanol per liter at pH 8.0. Material obtained from the crystals was dissolved in 75 μL of the same buffer. Samples were heated at 100 °C for 6 min; after they cooled, we added tracking dye (250 μL/mL), consisting of bromphenol blue, 0.7 g/L, in water/glycerol (2/1, by vol). The resulting solution (10 μL, whether derived from the crystals or the urine samples) was used for electrophoresis.

We electrophoresed the samples on 1-mm-thick, 8–20% gradient gels, using a Laemmli buffer system (30) with Bio-Rad Mini-Protein II apparatus. The stacking gel (6%) was formed with a 15-well comb. The protein bands either were detected with a dual-staining technique with silver (31) and Coomassie Blue R-250 or were electrophoretically transferred onto nitrocellulose for immunological identification as detailed below.

Western blotting. After electrophoresis, we equilibrated the gels for immunological characterization at 4 °C in transfer buffer (per liter, 25 mmol of Tris, 192 mmol of glycine, and 200 mL of methanol at pH 8.3) for 30 min. Proteins were transferred at 100 V for 1.5 h onto nitrocellulose (Trans-Blot; Bio-Rad Labs.) by the method of Towbin et al. (32) with Bio-Rad Mini Trans-Blot apparatus.

Immunochromatographic staining of nitrocellulose. Immunochromatographic staining was performed at room temperature. Nonspecific protein-binding sites were saturated by incubating the membranes for 16 h in TBST buffer (per liter, 10 mmol of Tris, 150 mmol of NaCl, and 0.5 mL of Tween 20 at pH 8.0) containing 60 g of skimmed milk (Diploma; Unilac, Dandenong, Victoria, Australia) per liter. With gentle agitation, the membrane was soaked in primary antiserum that, depending on the titer, had been diluted 200- to 1000-fold in TBST containing skimmed milk at 10 g/L. The second antibody, conjugated to alkaline phosphatase, was diluted 10 000- to 30 000-fold in TBST, and the membranes were developed with use of a Protoblot Immunoscreening System (Promega, Madison, WI).

Results

Electrophoresis Studies

Figure 1 illustrates the SDS–PAGE protein patterns obtained by the demineralization of crystals precipitated from female and male urines. Qualitatively, there was no discernible difference in the composition of the urines from each sex, the same protein bands being detected in each case. As anticipated, centrifugation and filtration of the urines caused an almost complete loss of THG. Several other urinary proteins were also less prevalent in the centrifuged and filtered urines, indicating that their concentrations were reduced by these procedures. Ultrafiltration removed almost all of the urinary macromolecules.

Centrifugation and filtration of the urine before crystallization had little effect on the qualitative protein content of the crystal extracts, other than to reduce the degree of streaking in these tracks. This suggests that cellular debris and THG may, at least in part, contribute to the streaking.

A comparison of the electrophoretic patterns of the proteins isolated from crystals induced in the same urines illustrates that most urinary proteins are not included in calcium oxalate crystals. However, one particular protein (bands designated α), with an Rf value corresponding to a molecular mass of ~30 kDa, was present in the crystal extracts in quantities far exceeding those of any others. Western blotting (see below)

Fig. 1. SDS–PAGE protein patterns obtained by the demineralization of crystals induced in pooled female urine (left) and in pooled male urine (right)

The patterns were obtained from W, whole urine; SF, spun (centrifuged) and filtered urine; and UF, ultrafiltered urine. To the right of each of these SDS–PAGE tracks are the protein patterns of the demineralized crystals induced in these respective urines: WC, SFC, and UFC. For the sake of clarity, the proteins THG, Alb (albumin), α1m (α1-microglobulin), and Apo A1 (apo-lipoprotein A-1) have been labelled only in the whole urine (W) of the left gel. Similarly, molecular mass standards (std) have been labeled only in the right gel.
demonstrated that this band was neither apolipoprotein A-I nor α1-microglobulin, common constituents of normal urine with \( R_f \) values equivalent to molecular masses of \( \sim 28 \) and 30 kDa, respectively. Because this protein appears to be a hitherto unidentified component of urine, we have called it crystal matrix protein, or CMP. Despite the fact that ultrafiltration quite clearly reduced the total urinary protein concentration, CMP was still evident in the extracts of the crystals generated in the ultrafiltered urines.

Although, as previously mentioned, the electrophoretic protein patterns of the crystal extracts from the male and female urines were qualitatively similar, CMP was far more prevalent in the crystals derived from the female urines than in those from their male counterparts. In addition, centrifugation and Millipore filtration of the urines before crystallization appeared to considerably diminish the quantities of this protein in crystals of male extract, although the reason for this is currently unknown. Furthermore, whereas the second most abundant protein in the crystals from the women’s urines had an \( R_f \) value corresponding to a molecular mass of approximately 43 kDa (protein band b), the next most predominant protein in the males exhibited a molecular mass of 66 kDa (bands designated c). Western blotting (see below) showed that neither of these was a common urinary protein.

**Western Blotting**

Using polyclonal antibodies, as detailed in the Methods section, we failed to demonstrate by immunoblotting that either immunoglobulins or their light or heavy chains, factor C3, α1-acid glycoprotein, α1-microglobulin, apolipoprotein A-I, β1-microglobulin, or fragmentation products of these major urinary proteins, were significant constituents of the crystal protein extracts, despite the fact that all of these proteins had been detected in the urine specimens from which the crystals were derived. Most noteworthy were the findings that THG, the major urinary macromolecular constituent, could not be detected in the crystals and that albumin, the next most plentiful protein, was present in only trace amounts. In contrast, the proteins that were most abundant in the crystals appeared to be only minor urinary constituents.

**Discussion**

The very presence of organic matrix throughout all renal calculi implies that this material fulfills a decisive role in stone formation and that it may direct the initial steps in the process, either as an inhibitor or a promoter. For this reason, the matrix has been the focus of considerable attention for many years. However, for the most part, previous findings must be regarded with a great deal of skepticism. The field has always been plagued with severe difficulties, including the refractory insolubility of the matrix, which has seriously hampered the identification of its components, and the fact that the isolation procedures used to extract and separate the organic constituents may have destroyed them or modified their chemical properties (3, 28). A further drawback is that the stones used for matrix isolation and characterization have often been in storage for many years before their analysis, such that their dehydration and aging in the intervening period will undoubtedly have resulted in chemical cross-linking and polymerization of component macromolecules (8). Urinary proteases and bacterial contamination would also affect the components of the matrix. Matrix isolated from such stones may therefore bear no chemical resemblance to the material that was originally occluded into the individual crystals composing the stone. On the other hand, none of these processes is likely to have occurred to any significant degree in newly formed crystals: determination of their organic constituents should therefore disclose more accurately the involvement of macromolecules in the initial crystallization process, free from the consequences of such secondary events.

Perhaps of most vital importance, however, has been the almost complete failure of any attempt to distinguish between organic material occluded *within* the crystals during the initial nucleation, growth, and aggregation of stone crystals and that deposited *superficially* as a consequence of trauma from various possible sources. Such events might include cellular injury induced by crystals simply abrading the urothelium, by the attachment of crystals to and subsequent release from renal cell membranes, or by the lodging of a stone in the kidney tract—a cycle of events that could be repeated as the stone grows in size. This distinction in the source of organic matrix is not trivial; it may hold the key to the function that matrix macromolecules fulfill in stone pathogenesis. And there is some evidence that such a distinction is justified.

By studying the organic matrix of calcium oxalate crystals freshly nucleated from human urine, and thereby overcoming the major difficulties associated with the study of stone matrix, Morse and Resnick (15) observed that urinary proteins are selectively associated with calcium oxalate crystals; i.e., many proteins present in urine could not be detected in crystals precipitated from it. In particular, albumin, the most abundant protein in their filtered urine samples, was present in the crystals only in diminished amounts or was absent altogether. Our results have substantiated both of these observations and, in addition, have demonstrated that THG, the most plentiful macromolecule in human urine, is virtually undetectable in calcium oxalate crystals generated in its presence. We have previously reported that THG is a potent inhibitor of calcium oxalate crystal aggregation (20). Others have suggested that crystal aggregation is inhibited by the adsorption of protein to the crystal surface (10) and that real inhibitors retard interface phenomena by blocking active sites on the surface (39). Therefore, molecules that inhibit aggregation but have little, if any, effect on crystal growth should be included in crystals formed in their presence. Urinary macromolecules are known to exhibit such features (20, 27), being potent inhibitors of
calcium oxalate crystal aggregation in undiluted urine and having only a minor influence on the deposition of new crystalline material. Under these circumstances crystal growth would be expected to proceed on growth sites not coated by the macromolecules, which would then become embedded within the growing crystal. Such a mechanism is described by Nancollas and Gardner (34). Discussing proposals put forward by other authors to explain the inclusion of impurities into crystals as growth proceeds, they point out that the growth front will be forced to pass over the impurities if these have bound to plane regions between the growth steps on the crystal surface and that, as a consequence, the impurities will become buried into the crystal surface. Certainly in biological systems, stray impurities are known to be included in crystals, and in many cases proteins appear to be integrated into the crystal phase (35–37).

Our failure to detect THG in calcium oxalate crystals is therefore significant in view of its strong inhibitory influence on crystal aggregation in urine. It has been well documented that THG exists in an aggregated state in normal urine (16, 17) and that, consequently, urine should more correctly be regarded as a gel than as a true solution (18). Possibly, therefore, as we have suggested elsewhere (38), the inhibition of crystal aggregation by THG can be ascribed to the physical characteristics of this glycoprotein in urine, which by steric hindrance, rather than by physicochemical adsorption, may prevent newly formed single crystals from becoming close enough to allow aggregate formation.

The findings of this study and that of Morse and Resnick (15) are in direct contrast to the work of Leal and Finlayson et al. (10) in studies of simple inorganic solutions. The latter authors showed that, per gram, several urinary proteins have approximately the same adsorption energy and therefore the same binding affinity for calcium oxalate crystals. Although Finlayson et al. did not test the binding of THG, they expressed the view that urinary mucoprotein would exhibit the same behavior. Our observations and those of Morse and Resnick, therefore, highlight once again a problem that has been recognized in urolithiasis research for years: results of studies performed in inorganic solutions cannot be used to predict probable outcomes in undiluted urine or to assign roles to urinary components in stone pathogenesis.

In view of our findings and those of Morse and Resnick, the absence of albumin and THG from newly formed calcium oxalate crystals also reaffirms the likelihood that two types of matrix are involved in stone formation, in light of evidence (7, 11) that both proteins are found in the matrix of stones. Although neither protein has a strong binding affinity for the surface of calcium oxalate crystals in undiluted urine, small quantities of urine containing high concentrations of the molecules could superficially become trapped in the interstices between individual crystals that comprise a larger aggregate. Continuing deposition upon the growing stone of additional crystals precipitated from a supersaturated urine would result in the entrapment of both THG and albumin throughout the final stone architecture.

Perhaps the most significant result of the present study was the detection of a protein (molecular mass ~30 kDa) within the crystals in quantities disproportionately greater than its concentration in urine. The presence and incongruous abundance of this protein was also noted by Morse and Resnick (15). These authors tentatively identified the protein as a2-microglobulin, but have since acknowledged that it should have been described as a1-microglobulin (personal communication), an immunosuppressive serum protein present in miniscule concentrations in normal urine and a marker for tubular proteinuria. However, using Western blotting, we were unable to detect a1-microglobulin as a major component of the crystals. Nor could we, using polyclonal antibodies, identify the predominant crystal protein as any of the major urinary constituents or as a fragmentation product of them. We conclude, therefore, that the protein is a minor urinary constituent possessing a remarkable binding affinity for calcium oxalate crystals. In the absence of any positive identification, we have called the protein crystal matrix protein. Its broad electrophoretic pattern is suggestive of a glycoprotein (39)—a consequence of various degrees of glycosylation after translation, which results in a variety of apparent molecular masses.

Although there was no qualitative difference in the protein composition of the male and female urines, it was particularly interesting that we observed a far higher concentration of CMP in the crystals derived from the urine from women than from men. Given that the incidence of urolithiasis is far lower in the female population and that women’s urine specimens reportedly inhibit calcium oxalate crystal aggregation more potently than do men’s (25), it is tempting to speculate that women’s relative protection from urolithiasis may be a consequence of an increase in amount or inhibitory activity of CMP in their urine. This notion is particularly attractive in view of other studies in progress in our laboratory that show CMP to be a potent inhibitor of calcium oxalate crystal aggregation, both in inorganic solutions and in undiluted urine. Because this finding has ramifications far beyond simply providing an explanation for the difference in stone incidence between the sexes, further studies are aimed at examining the possibility that CMP may serve a determinant role in the pathogenesis of calcium oxalate stone formation.

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