Effect of prothrombin and its activation fragments on calcium oxalate crystal growth and aggregation in undiluted human urine in vitro: relationship between protein structure and inhibitory activity

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ABSTRACT

In recent years there has been great interest in the putative role of prothrombin and its activation peptides, especially the urinary form of prothrombin fragment 1, in the pathogenesis of calcium oxalate (CaOx) urolithiasis. Previously, we showed that prothrombin and its activation peptides inhibit CaOx crystallization in inorganic conditions in vitro. The aim of the present study was to determine if this inhibitory activity is retained in undiluted human urine and, therefore, whether it is likely to have any influence under physiological conditions. A secondary objective was to assess the relationship between the structures of the proteins and their inhibitory activities. Prothrombin was purified from Prothrombinex-HT, cleaved with thrombin and the resulting fragment 1 (F1) and fragment 2 (F2) were purified. The purity of each protein was confirmed by SDS/PAGE, and their effects on CaOx crystallization in undiluted ultrafiltered human urine were determined at a final concentration 80.65 nmol/l using Coulter Counter and [14C]oxalate analysis. The precipitated crystals were visualized using scanning electron microscopy. The Coulter Counter data revealed that, whereas prothrombin and its activation peptides did not affect the urinary metastable limit and the size of the precipitated particles, F1 did significantly reduce the latter. These findings were corroborated with scanning electron microscopy which also revealed that the reduction in particle size caused by F1 resulted from a decrease in the degree of crystal aggregation, rather than in the size of the individual crystals. The [14C]oxalate data showed that none of the proteins added significantly inhibited the mineral deposition. It was concluded that with the exception of F1, which does inhibit CaOx crystal aggregation, prothrombin and its activation peptides do not alter the deposition and aggregation of CaOx crystals in ultrafiltered human urine in vitro. Also, the γ-carboxyglutamic acid domain of prothrombin and F1, which is absent from thrombin and F2, is the region of the molecules that determines their potent inhibitory effects. The superior potency of F1, compared with prothrombin, probably results from the molecule’s greater charge-to-mass ratio.

Key words: calcium oxalate crystallization, prothrombin fragment 1, urinary prothrombin fragment 1, prothrombin fragment 2, thrombin, urolithiasis.

Abbreviations: CaOx, calcium oxalate; Gla, γ-carboxyglutamic acid; THG, Tamm-Horsfall glycoprotein; UPTF1, urinary prothrombin fragment 1; HSA, human serum albumin; F1, prothrombin fragment 1; F2, prothrombin fragment 2; F1+2, prothrombin fragment 1+2.

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INTRODUCTION

All urinary stones are composed of an inorganic phase, which in the majority of cases is calcium oxalate (CaOx) [1], and a non-dialysable organic phase comprising principally protein, which is commonly known as matrix [2]. Although the proteinaceous component of stone matrix accounts for only a small percentage of a stone’s mass [2], its invariable occurrence in calculi [2–4] and distribution throughout the mineral phase [5] suggest that proteins may play some directive role in calculogenesis, either as promoters or incomplete inhibitors. To date, some 19 proteins have been identified in stone matrix and the list is ever increasing [6]. Despite this, all attempts to assign an unambiguous role to the proteins in stone matrix have proved fruitless. This is not surprising in view of the fact that proteins in stone matrix could have three different origins: some could be normal components of urine which promote or incompletely inhibit crystallization of the stone mineral; others could be released into the urine as a result of cellular damage caused by the growing stone; and the remainder could be adventitious adsorbants which play no active role in crystal formation [7].

Fortunately, the confusion caused by derivation of matrix proteins from several sources has been largely avoided by studying proteins isolated from CaOx crystals generated in healthy human urine, which does not contain macromolecules arising from epithelial trauma. A number of proteins associated with the organic matrix of such crystals have been identified [7–12]. However, the most abundant protein is a urinary form of the F1 activation peptide of prothrombin [13], now generally referred to as urinary prothrombin fragment 1 (UPTF1). The properties of UPTF1 suggest that it may play an important regulatory role in stone pathogenesis. It occurs in calcium stones [14], and is expressed in the distal convoluted tubules and thin ascending limbs of the loops of Henle of selected nephrons [15]. Its altered immunohistochemical staining in the kidneys of human stone formers [15], combined with the fact that it is a potent inhibitor of CaOx crystal growth and aggregation in undiluted human urine [16], and that its parent molecule, prothrombin, is also synthesized in the human kidney [17], reinforce the possibility that UPTF1 fulfils a protective function in urolithiasis.

However, the F1 activation peptide is not the only derivative of prothrombin which, if present in urine, could participate in CaOx crystallization. During blood coagulation, prothrombin in plasma is cleaved by activated Factor X to thrombin and fragment 1 + 2 (F1 + 2). After this initial scission, the released thrombin cleaves F1 + 2 to fragment 2 (F2) and fragment 1 (F1) [18]. It is widely believed that prothrombin is absent from human urine, except in patients with nephrotic syndrome or during so-called hypercoagulable states [19]. Nonetheless, we have routinely detected prothrombin and F1 + 2 in the urine of healthy men and women who do not have haematuria, as well as in CaOx crystals generated from them [20]. The presence in CaOx crystals of prothrombin itself and its two smaller fragments can be attributed to their binding to the crystal surface and subsequent incarceration within the crystal bulk, which is almost certainly mediated by the N-terminal domain common to them all. Containing ten residues of γ-carboxyglutamic acid (Gla), this region is responsible for effecting the stereospecific structural transitions required for attachment of prothrombin to the phospholipid membrane during the clotting of blood [21]. This is achieved by its high affinity for Ca2+ ions [22], a property that undoubtedly accounts for the incorporation of prothrombin and its F1 + 2 and F1 fragments into urinary CaOx crystals. This raises the possibility that they might also have similar inhibitory effects on CaOx crystal growth and aggregation. This inference is supported by the observation that in an inorganic medium, prothrombin inhibits the growth of hydroxyapatite [23], which, after CaOx, is the most common calcium salt in urinary calculi [1].

However, it is possible that the remaining two derivatives of prothrombin, namely the F2 and the catalytic thrombin moiety, neither of which possess the Gla domain, may be also capable of participating in CaOx crystallization by binding to the crystal surface via other molecular regions. Although F2 has been identified in human urine [24], thrombin could not be detected by immunological means. This is puzzling, since the polyclonal anti-thrombin antibody used in that study should have cross-reacted with prothrombin, which along with the non-thrombin portion of the molecule, F1 + 2, are commonly observed to be present in urine [20]. As far as we are aware, no later study has attempted to measure thrombin in human urine; its presence in urine cannot therefore be discounted.

Because F1, F2 and thrombin represent contiguous, but distinct regions of their parent, which together comprise the entire primary structure of the prothrombin molecule, with no duplications or omissions of its amino acid sequence [25], they represent a unique means to study the relationship between protein structure and inhibition of CaOx crystallization. We have used previously a similar strategy to examine the effects of the same moieties of the prothrombin molecule in a seeded inorganic crystallization system [26]. An inorganic model was used because such systems require much smaller quantities of proteins than are needed to assess inhibitory activity in urine itself, and the availability of pure preparations of each protein were limited. However, it is widely acknowledged that the effects of modulators of crystallization in inorganic media cannot be extrapolated to predict any influences they may have in urine itself [27]. Thus the first aim of this study was to determine
whether prothrombin itself and its activation peptides, F1, F2 and thrombin, affect the deposition and aggregation of CaOx in undiluted ultrafiltered urine. The second aim was to determine whether the effects of each of the proteins could be related to their molecular structures.

METHODS

Chemicals
Benzamidine hydrochloride, tosyl-lysylchloromethane (‘TLCK’), tosylphenylalanylchloromethane (‘TPCK’), PMSF and EGTA were purchased from Sigma (St. Louis, MO, U.S.A.). [\(^{14}\)C]Oxalate was obtained from NEN Products (Boston, MA, U.S.A.). All other chemicals used in this study were of the highest purity available.

Purification of prothrombin, thrombin, F1 and F2
This has been described in detail previously [26].

SDS/PAGE
The purified lyophilized preparations of prothrombin, thrombin, F1 and F2 were reduced with 2-mercaptoethanol, and electrophoresed on 10–20% linear gradient gels (1 mm \( \times \) 8 cm \( \times \) 7 cm) on a Bio-Rad Mini-Protean II apparatus as described previously [7]. The gels were subsequently stained with silver [26].

Preparation of protein solutions
Purified prothrombin, thrombin, F1 and F2 were separately dissolved in distilled water and dialysed against phosphate buffer (pH 7.3), containing 8.5 g/l NaCl, 1.43 g/l K\(_2\)HPO\(_4\) and 0.25 g/l KH\(_2\)PO\(_4\), at 4 °C for 36 h. Protein concentrations in the samples were determined using the Bio-Rad protein assay with BSA (Sigma) as the standard protein.

Collection and preparation of urine samples
Urine passed in a 24-h period was collected from ten healthy men who had no history of kidney stone disease. The samples were refrigerated during the collection period and during storage before use. Absence of blood from the specimens was confirmed using Multistix test strips (Miles Laboratories, Mulgrave, Australia), and the samples were pooled. The pH of the pooled urine was 6.21. The pooled sample was then centrifuged using a JA-14 fixed-angle rotor at 8000 g for 15 min at 20 °C in a Beckman J2-21 M/E centrifuge (Beckman Instruments, Palo Alto, CA, U.S.A.). The supernatant was filtered through 0.22 μm Millipore filters (GVWP 14250; Millipore, Bedford, MA, U.S.A.), and a portion was retained as a control (centrifuged and filtered sample). This control contained its full complement of macromolecules apart from Tamm-Horsfall glycoprotein (THG), which is known to be removed from urine by centrifugation and filtration [28,29]. The remaining urine was ultrafiltered (nominal molecular mass cut off of 10 kDa) using an Amicon hollow fibre bundle (H1P10-20; Amicon, Lexington, MA, U.S.A.), and divided into five portions: one portion was used as an ultrafiltered control and the remaining four aliquots were supplemented with prothrombin, thrombin, F1 and F2 respectively. The effects of the proteins on CaOx crystallization were studied at a final concentration of 80.64 nmol/l. Molecular masses of the proteins used in this calculation were: prothrombin, 72.5 kDa; thrombin, 37 kDa; F1, 31 kDa; F2, 19.5 kDa [30]. This value was chosen because it is close to the physiological concentrations of activation peptides of prothrombin in urine [24], and a similar concentration of UPTF1 (2.5 mg/l or 80.65 nmol/l) was reported previously to inhibit significantly CaOx crystal growth and aggregation in a similar system of CaOx crystallization [16].

Measurement of crystallization by Coulter Counter analysis
This was assessed as described previously [16]. The metastable limit of each urine specimen was first measured by titration with sodium oxalate solution, a standard load of oxalate (30 μmol/100 ml) in excess of this amount was added dropwise to the samples. Because the minimum amount of oxalate required to induce detectable spontaneous CaOx crystal nucleation was unaltered by ultrafiltration of the urine and by addition of any of the proteins, the oxalate load was identical in all cases. Samples were then incubated in a shaking water bath at 37 °C for 2 h. Crystal size distributions were measured at intervals of 30 min using a TAII Coulter Counter fitted with a Population Count Accessory and a 70 μm orifice tube, and from these were calculated the total volumes of material precipitated. The size of the particles precipitated from the various urine samples were expressed as the modes of the volume distribution curves at the end of 120 min incubation period. Because the quantities of purified proteins were extremely limited, each experiment was performed only in duplicate. The results are presented as the mean of the two measured values.

Preliminary experiments revealed that the intra-assay and inter-assay coefficients of variation of measurement of crystallization by Coulter Counter analysis were 6.1 % and 8.9 % respectively, and that none of the proteins tested precipitated during the course of the experiment. Therefore control flasks containing all components other than oxalate were not included to account for possible counting interference caused by precipitated protein.
Scanning electron microscopy
At the end of each experiment, 2 ml aliquots of each sample were filtered (0.22 μm) and the filtration membranes were dried overnight at 37 °C. They were then mounted on to aluminium stubs, sputtered with gold for 180 s (SEM Autocoating Unit E5200, Polaron Equipment Ltd, Watford, U.K.), and examined using an ETEC Auto Scan Electron Microscope (Siemens AG, Karlsruhe, Germany) at an operating voltage of 20 kV.

Measurement of CaOx deposition by [14C]oxalate analysis
The use of the Coulter Counter to measure particle volume in the type of experiment described in the present study has well-documented limitations [16]. In summary, the Coulter Counter measures particles whose diameters fall within a specified range (in these experiments 2.0–25.4 μm); crystals with sizes outside this range will not be counted. Furthermore, loose aggregates of crystals containing empty spaces are recorded by the instrument as if they are solid, thereby giving erroneously high estimates of crystal volume deposition. This can be further compounded by the inclusion of macromolecules into the crystalline architecture. The Coulter Counter cannot account for differences in particle density. Therefore, to determine the true extent of mineral deposition, incubations were carried out with samples that were identical with those described above, except that they were supplemented with [14C]oxalate (13.33 μCi/100 ml) before the addition of the sodium oxalate load to induce crystallization.

At 30 min intervals, 1.0 ml of each sample was filtered (0.22 μm) into 100 μl of concentrated HCl using disposable syringes fitted with a filter holder (Sartorius Minisart NML, Gottingen, Germany). Duplicate 0.3 ml aliquots of the acidic solution were then added to 10 ml Ready Safe scintillation fluid (Beckman Instruments Inc.) and counted for 5 min in a liquid scintillation counter (Beckman LS 3801 Liquid Scintillation System). As before, each experiment was performed in duplicate and the results are expressed as the mean values.

Preliminary experiments revealed that the intra-assay and inter-assay coefficients of variation of measurement of CaOx deposition by [14C]oxalate analysis were 3.9% and 5.1% respectively.

Statistical analysis
The results were analysed using the paired Student’s t test at an 0.05 level of significance.

RESULTS
Purity of proteins
Figure 1 shows an SDS/PAGE electrophoretogram of the preparations of prothrombin, thrombin, F1 and F2 used in the experiments. Prothrombin and F2 are visible as single bands indicating a high state of purity. Thrombin and F1 show very minor contamination with F2 and prothrombin respectively. For the purposes of this study,
all these preparations were used without any further purification.

**Effect of prothrombin, thrombin, F1 and F2 on particle size**

Figure 2 shows the size of the particles precipitated from the various urine samples, expressed as the modes of the volume distribution curves at the end of the 2 h incubation period. The overall particle size in the ultrafiltered control urine (17.4 μm) was slightly reduced to 17.2 μm in the presence of thrombin. F2 reduced the particle size to the same extent as prothrombin, the value being 15.1 μm in each case. Addition of F1, however, markedly reduced the particle size to 8.9 μm, even smaller than that recorded in the centrifuged and filtered control urine (9.4 μm), which contained most of its urinary macromolecules other than THG. These values show that, whereas the addition of thrombin, prothrombin and F2 did not significantly affect the size of the precipitated particles, F1 reduced it by 48.85% \((P \leq 0.05)\) compared with the ultrafiltered control to which no such proteins were added. These findings were corroborated in Figure 3, which showed low power scanning electron micrographs of material precipitated in

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**Figure 3** Scanning electron micrographs of the crystalline material deposited in the centrifuged and filtered (C & F) urine and in aliquots of the same urine which had been ultrafiltered (UF) and spiked with prothrombin (PT), F1, F2 or thrombin (T)

This showed that, whereas the addition of prothrombin, thrombin and F2 to the ultrafiltered urine did not significantly alter the size of the precipitated particles, F1 reduced it quite dramatically. This reduction in the particle size, caused by the addition of F1 to the ultrafiltered urine, was due to a decrease in the degree of crystal aggregation, rather than in the size of individual crystals.

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**Figure 4** Percentage decrease in soluble [14C]oxalate with time after the addition of oxalate load in the centrifuged and filtered (C & F) urine and in aliquots of the same urine which had been ultrafiltered (UF) and enriched with prothrombin (PT), F1, F2 or thrombin (T)

The values show that prothrombin and its activation peptides, F1, F2 and thrombin, did not significantly alter deposition of CaOx compared with the ultrafiltered control to which no such proteins were added.
Table 1  Change in unprecipitated [¹⁴C]oxalate following the addition of oxalate in centrifuged and filtered (C & F) urine, and in aliquots of the same urine that had been ultrafiltered (UF) and spiked with prothrombin (PT), F1, F2 or thrombin (T).

<table>
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<th>Time (min)</th>
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The centrifuged and filtered and ultrafiltered urines, and in the samples containing the proteins. Mainly CaOx dihydrate crystals were deposited in all the urines: those from the centrifuged and filtered urine tended to be single or clustered into small loose aggregates, and were accompanied by organic material which was visible on the filtration membrane. In contrast, the crystals precipitated from the ultrafiltered urine were highly aggregated and there was no evidence of organic material on the filter surface. Both particle size analysis and scanning electron microscopy showed that, whereas the addition of prothrombin, thrombin and F2 to the ultrafiltered urine did not significantly alter the size of the precipitated particles, addition of F1 reduced it quite dramatically, and this reduction in the particle size resulted from a decrease in the degree of crystal aggregation, rather than in the size of individual crystals.

**[^C]oxalate measurement of CaOx deposition**

Figure 4 and Table 1 show the time course of the disappearance of [¹⁴C]oxalate following the addition of the oxalate load to the various urine specimens. The results were normalized by expressing values as the percentage of [¹⁴C]oxalate remaining in solution relative to that recorded at zero time. The rate of disappearance of [¹⁴C]oxalate from centrifuged and filtered urine was higher than in any of the ultrafiltered urines, either alone or in the presence of any of the proteins. Moreover, among the ultrafiltered urines supplemented with proteins, the rates of disappearance of [¹⁴C]oxalate were very similar. At 120 min, the mean [¹⁴C]oxalate remaining in the centrifuged and filtered urine control (63.1 %) was slightly less than in the ultrafiltered urine control (64.3 %), and also in the presence of added prothrombin (65.8 %), thrombin (67.0 %), F1 (64.4 %) or F2 (64.0 %). These values show that prothrombin and its activation peptides, F1, F2 and thrombin, did not significantly alter deposition of CaOx in relation to the ultrafiltered control to which no such proteins were supplemented.

**DISCUSSION**

The invariable association of organic material with crystalline CaOx in kidney stones [2–4], together with the inhibitory effects of a number of urinary proteins on CaOx crystallization (reviewed in [31, 32]), has reinforced the long-held view that proteins may perform regulatory functions in the prevention and/or formation of urinary calculi. Identification and characterization of natural urinary candidates for such a role are important, since they have the potential to assist our understanding of calculus formation and therefore open possible avenues for preventing stone recurrences.

One protein which may fulfil such a role is UPTF1, whose presence in urine could result directly from the renal synthesis of prothrombin [17] and/or the glomerular filtration of F1, resulting from turnover of the protein circulating in plasma. Although it is a widely held view that prothrombin itself is present in human urine only in certain disease states [19], we have frequently detected it in the urine of healthy male and female subjects who do not have haematuria and in CaOx crystals precipitated from it, although in far lower quantities than UPTF1 [20]. The fact that prothrombin contains the entire primary sequence of UPTF1 is sufficient to suggest that it too would be an efficient inhibitor of CaOx crystallization, a supposition that we have confirmed using a seeded inorganic crystallization system [26]. Nonetheless, such inhibitory properties may well be irrelevant under physiological conditions where other high and low molecular mass components of urine, as well as numerous ions, may interfere with its stereochemical structure or compete for binding sites on crystal surfaces. The aim of the present work was to determine whether the inhibition of CaOx crystallization by prothrombin, thrombin and F2, shown previously in an inorganic medium [26], is conserved in undiluted urine. Despite the fact that the strong inhibitory effects of UPTF1 in undiluted urine are well documented [16], F1 was also tested, since F1, F2 and thrombin, collectively account for the entire sequence of the prothrombin molecule [25], and therefore offer a unique opportunity to relate inhibitory activity to specific molecular regions of a single protein.

Isolation of prothrombin from Prothrombinex-HT yielded preparations of the protein that, following selective cleavage with thrombin, provided preparations of F1 and F2 whose purity was satisfactory for use in the inhibition assay system. In agreement with our previous studies [16, 33], neither ultrafiltration nor addition of any of the peptides affected the amount of oxalate required to induce detectable spontaneous nucleation of CaOx.
Identical amounts of oxalate were therefore added to each sample to induce precipitation, and any differences observed must therefore have resulted specifically from the effects of each individual protein.

Particle size is a well-recognized determinant of the likelihood of retention in the renal tubules, and hence of urinary stone formation. The significance of crystal aggregation cannot therefore be overstated, since it is the only process which can cause the formation of large, potentially dangerous particles during the short period of time urine is resident within the renal collecting system. Analysis of the Coulter Counter data revealed that, as always occurs [16,33], ultrafiltration increased the size of crystalline particles precipitated, with the average modal size of the particles deposited from the ultrafiltered urine being almost double those precipitated from the same urine that had been only centrifuged and filtered. Scanning electron microscopy confirmed that the augmentation in particle size resulted from an increase in aggregation, rather than from enlargement of individual crystals. With the exception of F1, which significantly reduced the size of the precipitated particles, this effect of ultrafiltration was not mitigated by the addition of prothrombin, F2 or thrombin. It is noteworthy that the proteins were derived from the same preparations as those used in a previous study [26] where their activities were tested in an inorganic reaction medium; as those used in a previous study [26] where their proteins were derived from the same preparations prothrombin, F2 or thrombin. It is noteworthy that the primary structures of these two proteins are identical and the reported differences in their staining patterns [13] are probably attributable to their derivation from serum or urine. In the absence of any other documented difference, the small disparity between the relative effects of UPTF1 and F1 almost certainly reflect differences in the chemical composition of the ultrafiltered urine samples in which their activities were measured, as well as possible heterogeneity in the degree of γ-carboxylation of their Gla domains. Isolation of the two closely related proteins from their respective sources may well result in uncontrolled decarboxylation of one or more of the Gla residues, which is most likely to affect the binding of each protein to the crystal surface and consequently its inhibitory activity. The only direct way to confirm these possibilities is to compare inhibitory activities of UPTF1 and F1 in the same ultrafiltered urine, and to perform two-dimensional gel co-electrophoresis analysis. Unfortunately, we have no UPTF1 remaining from our previous study [16].

As we consistently observe [26,37,38], the proteins inhibited CaOx crystal aggregation far more potently than they did solute deposition, and their effects in undiluted ultrafiltered urine were less than we have observed previously using an inorganic crystallization model [33,37,38]. In the present study, neither prothrombin itself, nor any of its activation fragments, materially altered the amount of CaOx deposited in relation to the ultrafiltered control urine, despite the fact that in a previous study [26] identical preparations of the same peptides significantly reduced mineral deposition in the order F1(44%) > prothrombin(27.4%) > thrombin(10.2%) > F2(6.5%) at a much lower final concentration (16.13 nmol/l). The disparity in the effects of the proteins between the two studies again cautions against extrapolating findings from inorganic solutions to predict likely effects in whole urine and, more particularly, on stone formation. The only other urinary protein whose effects on CaOx deposition have been measured in undiluted ultrafiltered urine, THG, did not affect CaOx deposition [38]. However, at high urine osmolalities induced to non-physiological levels by evaporation, THG markedly promoted CaOx deposition [38,39].

More important is the apparent discrepancy between the extents of reduction of mineral deposition wrought
by UPTF1 and F1 using the same experimental system. Previously, we showed that UPTF1 reduced $[^{14}C] \text{CaOx}$ deposition by 32.2% [16]. In the present study, the same concentration (2.5 mg/l or 80.65 nmol/l) of F1 did not measurably reduce crystal growth. When results for F1 from the present investigation are compared directly with those obtained for UPTF1 from our previous study [16], it is apparent that UPTF1 is a more potent inhibitor of crystal growth than F1, but a weaker inhibitor of crystal aggregation. In the absence of any incontrovertible evidence that UPTF1 and F1 have significant structural disparities which might explain their apparent discrepant effects, we must again conclude (see above) that the seemingly contradictory findings result from the fact that results were obtained using different ultrafiltered urine samples as the reaction medium, and possibly from de-$\gamma$-carboxylation of one or more of the Gla residues comprising the Gla-containing domains of the proteins. It is well known [21] that the action of prothrombin is compromised by de-$\gamma$-carboxylation of its constituent Gla residues, which reduces the protein’s ability to bind to phospholipid membranes during the coagulation of blood. There is little doubt that similar conversion of Gla to glutamic acid in both F1 and UPTF1 will also decrease their ability to bind irreversibly to the CaOx crystal surface and will alter their effects on growth and aggregation.

In addition to providing results regarding the effects of prothrombin and its cleavage products on CaOx crystallization, the present study has also yielded information about the relationship between molecular structure and inhibitory effects on crystal aggregation. F1 significantly inhibited crystal aggregation, in line with our previous study [26] attributing its effects to the Gla domain portion of the molecule, which undoubtedly mediates the binding of F1 to the CaOx crystal surface and confers its inhibitory potency. Prothrombin, thrombin, and F2 had no significant effect on crystal aggregation. The lack of any significant action by thrombin and F2 is not surprising, in agreement with previous findings reporting a lack of effect on CaOx crystal aggregation and the absence of the Gla domain from their structures [26]. It is, however, puzzling that prothrombin did not significantly inhibit, since it does contain the Gla domain. It therefore follows that the Gla domain alone does not determine binding affinity or inhibitory potency. The molecular mass of prothrombin (approx. 72.5 kDa) is more than double that of F1 (approx. 31 kDa) and its isoelectric point is higher [40]. Thus it is likely that the lack of effect of prothrombin may perhaps be attributable to its lower charge-to-mass ratio than F1.

It is interesting to note that the results of studies comparing concentrations of prothrombin and its Gla-containing fragments (F1 + 2 and F1) in the urine of healthy volunteers and of stone formers have been contradictory [41,42]. Since the authors of these studies [41,42] did not check the samples for the presence of crystals before analyses, this inconsistent finding could perhaps be attributable to varying extents of CaOx crystalluria. This is because Gla-containing domain of prothrombin and its fragments (F1 + 2 and F1) almost certainly causes the proteins to bind to CaOx crystals, formed endogenously or after excretion [43]. This effectively removes the proteins from the solution phase, therefore resulting in their underestimation in urine. This has significant ramifications, since measurements of proteins in urine are typically carried out on refrigerated and frequently frozen samples, for later batch analysis; cooling and freezing are well known to cause formation of CaOx crystals. This issue is further compounded as CaOx crystalluria is more common and more extensive in recurrent stone formers than in normal subjects [44]. Thus the potential for serious error is greatest in the very group in which the measurement of prothrombin and its Gla-containing fragments (F1 + 2 and F1) might impart the most benefit. It is remarkable that this problem is not confined only to prothrombin and its Gla-containing fragments (F1 + 2 and F1); doubts must now be raised about published results presenting excretion values for proteins such as bikunin [45,46], osteopontin [47,48] and, indeed, any protein implicated in urolithiasis that binds irreversibly to CaOx crystals. Further studies are required to design strategies for preventing CaOx crystal formation in urine prior to protein measurements for urolithiasis research or diagnostic purposes.

The results of the present study clearly demonstrate that, although prothrombin and its cleavage products do not inhibit CaOx deposition and aggregation in undiluted ultrafiltered urine, F1 does significantly reduce crystal aggregation and may therefore exert similar effect under physiological conditions. Also, although F2 did not significantly reduce crystal aggregation, its absence from CaOx crystal matrix [13] suggests that, though present in urine, it is unlikely to participate to any significant extent in CaOx crystallization in the urinary tract. Similarly, the superior inhibitory activity of F1, as well as its markedly higher urinary concentration than that of prothrombin, suggests that its contribution to the inhibitory potential of urine is likely to overwhelm any effects that prothrombin might have in vivo. This provides further indirect support for the possibility that the urinary form of F1 may fulfil a decisive role in the crystallization of CaOx in the urinary tract, and thereby in stone pathogenesis.

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