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INTER-ALPHA-INHIBITOR: A PROTEIN FAMILY INVOLVED IN THE INHIBITION OF CALCIUM OXALATE CRYSTALLIZATION

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Abstract

Inter-α-inhibitor (IαI) is a serine protease inhibitor present in human plasma. It has a molecular weight of about 220 kDa which encompasses 3 chains including two heavy chains and one light chain. The light chain, known as bikunin, is responsible for the antitryptic activity of IαI in the inhibition of various enzymes, such as trypsin and chymotrypsin. Under physiologic or certain pathologic circumstances, several macromolecules related to IαI appear in plasma and urine. However, the physiologic role of IαI remains unclear. As far as urolithiasis is concerned, two urinary macromolecules related to IαI have been isolated and shown to be potent inhibitors of calcium oxalate formation. One of these inhibitors, uronic-acid-rich protein (UAP), has been identified and well characterized. The sequence of the first 18 amino acid residues of UAP is identical with that of bikunin. Furthermore, the immunoreaction between UAP and IαI antibody using immunoblot analysis was positive. UAP isolated from the urine of stone formers exhibited less inhibitory activity towards calcium oxalate crystallization than that derived from the urine of healthy subjects. This suggests a structural abnormality of the inhibitor obtained from stone patients. The organic matrix extracted from kidney stones contained a protein antigenically related to IαI. We conclude that UAP is a member of IαI family taking part in inhibiting calcium oxalate crystallization, and modulating the formation of stones in the urinary tract.

Key Words: Urolithiasis, urine, plasma, calcium oxalate, inter-α-inhibitor, uronic-acid-rich protein, bikunin.

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Introduction

Mammalian blood is a rich source of proteinase inhibitors which account for about 10% by weight of all plasma proteins in human [46, 69]. They constitute the third largest group of functional proteins after albumin and immunoglobulins. Inter-α-inhibitor (IαI) is one among the well characterized human serine plasma proteinase inhibitors. These inhibitors seem to control diverse critical events associated with proteolysis that occur during pathologic circumstances such as cancer, fibrinolysis, and inflammation by inhibiting the action of various enzymes including neutrophil elastase, cathepsin G, and plasmin [20, 28, 50]. Nevertheless, the physiological function of IαI is still not well established. The average concentration of IαI in plasma of healthy human subjects is about 450 mg/l [65]. Low amounts of other smaller macromolecules immunologically related to IαI are also present in plasma and urine. Their level increases in pathological conditions, suggesting that they could be a result of an increase of IαI turnover. Many of these macromolecules have been purified and characterized to understand the mechanism of their release and the importance of their presence in the body fluids. A few years ago, two macromolecules related to IαI and having the ability to inhibit calcium oxalate (CaOx) crystallization *in vitro* were isolated from human urine [2, 63].

This paper is written to focus attention on the relationship between IαI and urolithiasis. We describe here IαI and its related proteins, specifically those that may be involved in stone formation.

Structure of IαI and its Immunologically Related Derivatives (Figure 1)

IαI is a glycoprotein with a molecular weight of about 180-220 kDa. A decade ago, it was thought that IαI contained a single polypeptide chain since it showed a unique band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of strong reducing agents [41, 51]. However, subsequent

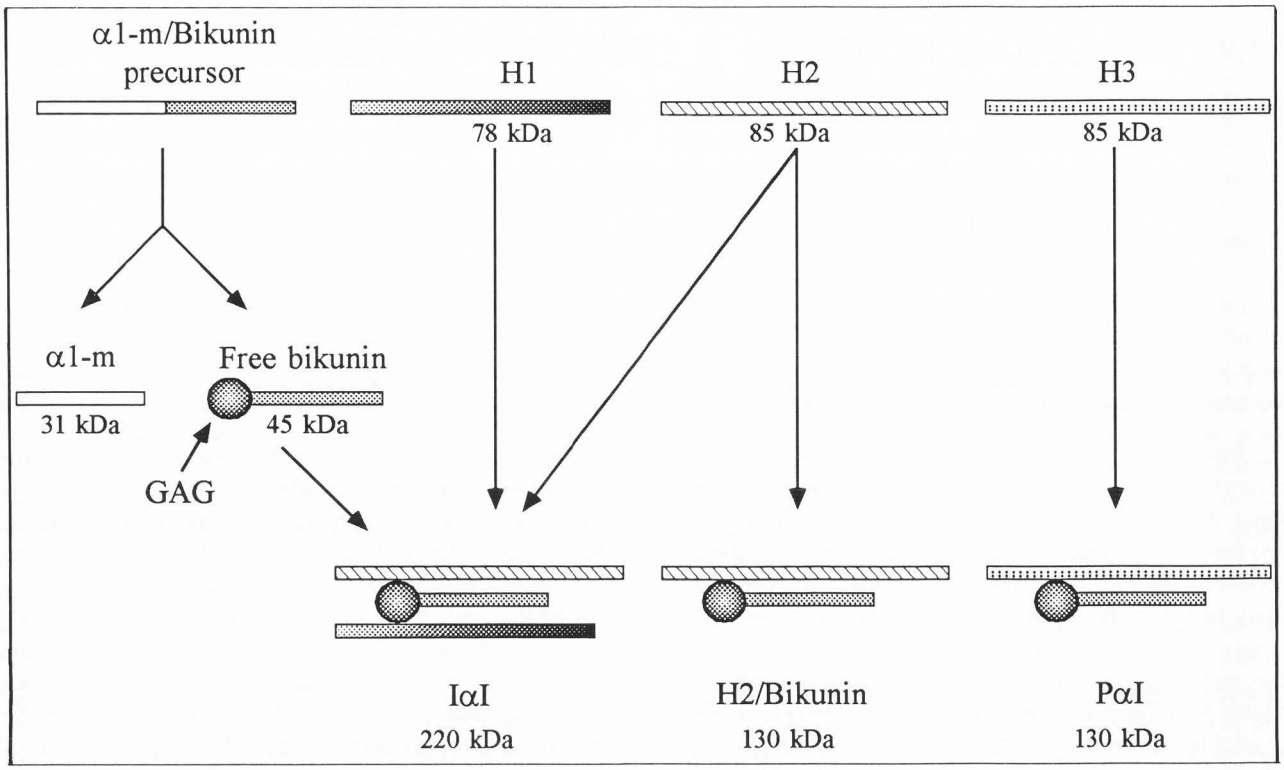

Figure 1. Structure of IαI and its derivatives according to reference 54. Bikunin is derived from a common precursor that codes also for α1-microglobulin (α1-m). It is present in a free form or complexed with one or two heavy (H) chains constituting H2/Bikunin, inter-α-inhibitor (IαI) (H1 + H2 + bikunin), or pre-α-inhibitor (PαI) (H3 + bikunin). A glycosaminoglycan (GAG) chain, associated with bikunin, ensures the junction between the different subunits of IαI and its related proteins.

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data coming from molecular biology techniques demonstrated the presence of two or three distinct mRNAs coding for heavy (H) and light (L) chain respectively [11, 19, 25, 56, 60]. The latter is called bikunin, formerly known as urinary trypsin inhibitor, acid-stable trypsin inhibitor, the light chain of IαI, or HI-30. Bikunin originates from a common mRNA that also codes for another protein called α1-microglobulin (α1-m) [34, 37, 53, 62, 71]. As soon as they are synthesized, a cleavage occurs and both α1-m and bikunin are released separately [49]. α1-m has never been found associated with IαI or its related polypeptides [56, 60]. In humans, the scission seems to occur at the amino acid sequence Arg-Val-Arg-Arg immediately preceding the bikunin subunit of the precursor [13]. Therefore, these amino acid residues are missing in the final product. Yet, the mechanism and conditions under which the precursor is broken away remain elusive. Now, it is recognized that IαI comprises two heavy chains (H1: 78 kDa and H2: 85 kDa) and bikunin (45 kDa) [10, 38, 45, 52]. The genes of these chains are located on the chromosome 3p21.1-p21.2, 10p14-p15, and 9q32-q34 respectively [19, 59]. Bikunin is also present in a free form in plasma and urine [54]. Interestingly, the three chains are held together in unique complex by way of a carbohydrate chain [32, 38]. Bikunin contains a *N*-linked oligosaccharide chain and an *O*-linked oligosaccharide chain attached to Asn^{45} and Ser^{10} respectively [25, 29]. The latter was further demonstrated to be a glycosaminoglycan (GAG) [17]. Several studies demonstrated that chondroitinase and hyaluronidase can split IαI into heavy and light chains [8, 9, 61]. Recent results confirmed that the GAG chain covalently cross-linked the subunits of IαI [40].

Another protein related to IαI has also been found and named pre-α-inhibitor (PαI) [22]. It is made of bikunin, similar to that of IαI and a heavy chain (H3: 85 kDa) coded by another mRNA different from those described earlier for IαI. This demonstrated that PαI constituted a protein distinct from IαI and not derived from it by proteolysis [22]. A protein consisting of H2 and bikunin is also present in the plasma [18, 21, 54]. The nature of the cross-linking between the two polypeptide chains of this protein appears similar to that found in

PαI. In all polypeptide complexes, IαI, H2/bikunin, or PαI, bikunin seems to be responsible for their antitryptic activity by inhibiting various enzymes, particularly trypsin and chymotrypsin [20, 28, 50].

When IαI and bikunin are submitted to a prolonged digestion with trypsin, they release fragments of a molecular weight of 14 and 8 kDa named HI-14 and HI-8, respectively [30, 72]. The amino acid composition of bikunin and HI-14 are identical indicating that HI-14 is the peptidic chain of bikunin and large carbohydrate moieties should be responsible for the molecular weight of 45 kDa of the native form of bikunin. Therefore, HI-14 and HI-8 may be products of IαI or bikunin which are fragmented during purification or in the presence of enzymes like trypsin.

Biosynthesis and Localization of IαI and its Derivatives (Table 1)

IαI is synthesized mainly in the liver and excreted in plasma [10, 62]. This finding was confirmed at the molecular level. Indeed, the mRNA encoding for the IαI subunits was found expressed only in the liver [56]. Nevertheless, the expression of the gene encoding for the heavy chain H3 has also been observed in the brain [55]. By using dot blot analysis, the mRNA encoding for the precursor α1-microglobulin/bikunin was found in rat at high level in the liver and kidney, low in the brain and testis [33]. Further studies by the same authors however, confirmed its synthesis in the liver only [37]. In pig, the expression of mRNA of this precursor was observed at high level in the liver and, at a low level, in the stomach [67].

The immunochemical distribution of IαI and its derivative fragments in different organs has been investigated (Table 1). Under normal conditions, IαI immunoreactivity was found in the liver, kidney, testis, gross intestine, cutis and brain [15, 73, 74]. Surprisingly, the immunoreaction was also detected in cultured fibroblasts [14] and human mast cells [44]. Also using α1-microglobulin antibody, a positive immunoreaction was observed in human lymphocytes and macrophages [33]. The presence of IαI related proteins in the urinary tract has been specially investigated [44]. IαI immunoreactivity was found to be exclusively present in renal proximal tubules due to the reabsorption of bikunin by this segment of nephron. IαI immunoreactivity was also detected in human mast cells in the connective tissue of a bladder papilloma and in the rat bladder epithelium [44].

Table 1. Distribution of IαI and its derivatives in normal tissues.

Type of tissue	Method	References
Liver	Immunohistology	[15]
	mRNA analysis	[37*, 55, 56, 67*]
Lung	Elisa	[15]
	Immunohistology	[73]
Kidney	Immunohistology	[15, 44, 73, 74]
Brain	Immunohistology	[15, 73, 74]
Stomach	Immunohistology	[73, 74]
	mRNA analysis	[67*]
Testis	Immunohistology	[15]

*Distribution of α1-microglobulin/Bikunin mRNA

Physiological Role of IαI and its Related Proteins

The presence of IαI in various tissues suggests that it may fulfill different physiological functions. So far, however, these roles have not been resolved. IαI is especially sensitive to some enzymes such as elastase and cathepsin G which are activated during several diseases including cancer, inflammation, and renal failure. Due to their smaller size, proteins obtained from IαI turnover may be able to diffuse throughout the affected tissues and may contribute to the cells' protection against proteolysis. Nevertheless, IαI accounts for less than 5% of the total trypsin inhibitory activity [6, 57] which does not favor such a protective role. Moreover, the results showed no significant consumption of IαI during the inflammation process, although proteins related to IαI were present [58]. However, these results were contested in other studies [26, 45].

McKeehan *et al.* [39] isolated from human hepatoma cells two endothelial cell growth factors structurally similar to pancreatic secretory trypsin inhibitor and bikunin. Both proteins are absent or present in low amounts in normal plasma and urine. They stimulate endothelial cell growth and their concentrations were increased during inflammation and cancer. These findings suggest that IαI and their related proteins may be involved in neovascularization and desmoplasia, associated with tumor and tissue damage process.

Lastly, Sørensen's work [63] and particularly our studies [2] demonstrated that a glycoprotein related to IαI, most probably a urinary bikunin, is a potent inhibitor of CaOx crystal growth *in vitro* and may inhibit and reduce the formation of CaOx crystals in urinary tract.

Relationship of IαI and its Derivatives to Urolithiasis

Sørensen *et al.* [63] isolated a protein from normal human urine using two anion exchange chromatography

Table 2. Comparison of physico-chemical properties of Sørensen's protein, uronic-acid-rich protein (UAP), and urinary bikunin.

Proteins	Molecular weight (kDa)	CaOx growth inhibitory activity	Effect of pronase on CaOx inhibition	Effect of chondroitinase
Sorensen's protein	40	+++	No effect	Uronic acid removed
UAP	35	+++	Activity destroyed	Produces a protein of 20 kDa
Urinary bikunin	45	+	Activity destroyed	Produces a protein of 20 kDa

steps (on DEAE-Sephacel and Mono Q column) and one affinity chromatography (vinylsulfone agarose). The protein exhibited quite the same characteristics as urinary bikunin: molecular weight in SDS-PAGE: 40 kDa, in gel filtration: 67 kDa, identical N-terminal sequence, and presence of uronic-acids. The authors found that this IαI fragment was a potent inhibitor of CaOx crystal growth and stated that it is the only protein which might have an inhibitory activity in the urine. By chondroitinase digestion, the uronic acids were removed but the inhibitory capacity of the protein remained. Moreover, pronase digestion had no effect upon its inhibitory capacity and the authors considered that this data might be explained by the antiprotease activity of the inhibitor [63]. Unfortunately, further characterization of this inhibitor has so far not been reported.

In an attempt to find another CaOx inhibitor, we fractionated human urinary macromolecules using three chromatographic procedures including DEAE-Sephacel gel followed by Sephacryl S-300 chromatography, and finally Mono Q column using an FPLC system. We were able to isolate and purify a macromolecule with a molecular weight of 35 kDa as estimated by SDS-PAGE [2]. The protein showed a potential activity to slow the rate of CaOx crystal growth efficiently. Moreover, as described in detail in reference [2], its crystallization inhibitory activity was greater than that of nephrocalcin or nephrocalcin-like macromolecule considered, at the time, the principal urinary inhibitor of CaOx crystallization [42]. The inhibitor was named uronic-acid-rich protein (UAP) due to its uronic acid contents. The molecular characteristics of UAP indicated structural similarities with bikunin. The sequence of the first 18 amino acid residues of the protein was identical with that of bikunin [1, 4]. Moreover, by using a polyclonal IαI antibody by immunoblotting analysis, a positive immunoreaction was observed confirming the close relationship between UAP and IαI [1].

UAP was also isolated from rat urine and compared to that isolated from human urine [1]. Both showed identical amino acid composition, similar amino acid sequence, and similar crystallization inhibitory activity. UAP was assayed on CaOx crystal growth model before and after treatment with pronase, chondroitinase AC, and hyaluronidase as described by Sørensen *et al.* [63]. Our data demonstrated that chondroitinase and hyaluronidase had no effect on CaOx growth inhibitory activity of UAP, but yielded a protein with a molecular weight of 20 kDa [4]. This finding suggests that UAP comprises a large glycosaminoglycan chain responsible for its molecular weight at 35 kDa. In contrast to Sørensen's study, pronase treatment degraded the total inhibitory activity of UAP suggesting that the inhibitor isolated by us is different from the one purified by Sørensen *et al.* [63]. A comparison of physico-chemical proprieties of these proteins is shown in Table 2. We hypothesize that both UAP and Sørensen's protein may exist in urine as separate entities or they may be the same protein purified by slightly different methods.

Is UAP Bikunin?

The results of amino acid sequence and immunoblotting analysis suggest that UAP is a protein related to IαI family and could be bikunin. To clarify this relationship, in our recent study (Atmani *et al.*, Eur. J. Biochem., in press), both UAP and urinary bikunin were isolated and purified from human urine. Their CaOx growth inhibitory activity was assayed before and after pronase treatment. The inhibition assay consists in using a mixture of 1 ml supersaturated solution of $CaCl_2$ (2 mmol/l in Tris 0.05 mol/l, NaCl 0.15 mol/l, pH 7.3) in the presence of $[^{45}Ca]CaCl_2$ and 1 ml ammonium oxalate (2 mmol/l in Tris 0.05 mol/l, NaCl 0.15 mol/l, pH 7.3). The tubes containing proteins were compared with those without added proteins. At the end of the assay, tubes were centrifuged at 2000 *g* for 5 minutes and 0.5 ml of supernatant was withdrawn for radioactivity determination in a liquid scintillation counter. The inhibitory activity was determined before and after enzymatic digestion. The results shown in Figure 2 demonstrate that UAP exhibited a strong CaOx growth inhibitory activity while urinary bikunin was less inhibitory. The CaOx inhibitory activity of both proteins disappeared after pronase digestion. Structurally, UAP and urinary bikunin have many similarities. Their molecular weight

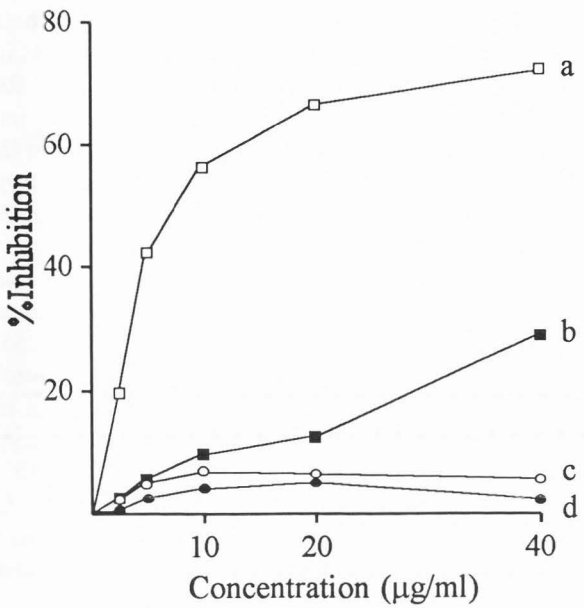

Figure 2. Calcium oxalate growth inhibitory activity of UAP and urinary bikunin before (**a**, **c**) and after treatment (**b**, **d**) with pronase respectively.

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is 35 and 45 kDa respectively. They have similar amino acid composition and similar amino acid sequence of first 18 residues. Furthermore, they are immunologically related to IαI. However, functionally they appear different. A comparison of physico-chemical proprieties of UAP and urinary bikunin is reported in Table 2.

As we have reported above, bikunin is present in free form or attached to different complexes related to IαI. The free form can easily pass into urine through glomeruli due to its low molecular weight. Metabolism of IαI or its related proteins (enzymatically for instance) may yield another form of bikunin. The latter may be modified during this process and present some differences compared to the preexisting free form of bikunin. Furthermore, the isolation of bikunin with different molecular weights, has been reported by several investigators [7, 9, 16, 23, 61]. These discrepancies may be due to differences in various technical procedures used in the isolation and purification of proteins. The carbohydrate content of the protein and the presence of a GAG chain that can modify its electrophoretic mobility should be taken into consideration since many results are still in disagreement. However, it has been reported that the carbohydrate chain does not contribute to CaOx inhibitory activity of proteins related to IαI [2, 63]. Experiments are in progress to clarify this matter.

Nephrocalcin with a molecular weight of about 14 kDa, is one of the well-known urinary inhibitors of CaOx crystallization [42]. So far, the amino acid sequence of this inhibitor remains unknown. We isolated a human urinary protein which we named nephrocalcin-like [2] due to its biochemical similarities with nephrocalcin described by Nakagawa *et al.* [42]. The molecular weight of the protein was about 16 kDa, it was able to bind to Tamm-Horsfall protein, and it showed an important inhibitory activity toward CaOx crystallization. However, this inhibitory activity was lower than that of UAP. Unfortunately, at that time, we did not determine its amino acid sequence. In a recent study, Ryallš group [66] reported the partial amino acid sequence of a protein claimed to be nephrocalcin. It showed an identity with HI-14, a fragment of bikunin. As we mentioned earlier, prolonged digestion of IαI or bikunin with trypsin, results in the production of two peptides, HI-14 and HI-8. Consequently, HI-14 is not a physiological product. Moreover, many features of nephrocalcin do not match with HI-14 fragment. For instance, nephrocalcin has 2-3 γ-carboxyglutamic acid (Gla) residues and is synthesized in kidney, but HI-14 does not contain Gla and is not produced in kidney. Therefore, the results obtained by Ryallš group remain speculative.

IαI and its Related Proteins in Lithiasis Disease

Many reports indicate that urine of stone formers contains macromolecules that inhibit CaOx crystal formation, but not efficiently as the same inhibitors found in the urine of normals [3, 27, 43]. With regards to IαI, its plasma concentration was reduced during various pathologic conditions including renal failure [24, 45, 46, 68]. Simultaneously, urinary bikunin concentration was increased suggesting an enhancement of IαI turnover. Although, these findings were not confirmed in other studies suggesting that IαI and its related proteins could be synthesized in separate pathways [13, 47, 58]. Accordingly, since UAP is related to IαI, may be its excretion is increased in the urine of patients with renal disease. Thereby, an increase in CaOx growth inhibitory activity is anticipated. Unfortunately, this was not the case, suggesting that UAP obtained from stone formers may be structurally abnormal and does not inhibit crystal precipitation efficiently. Accordingly, we have isolated UAP from the urine of stone formers and its inhibitory activity was compared to that purified from the urine of healthy subjects [3]. The results demonstrated that UAP obtained from stone patients exhibited less inhibitory activity. Partial structure of this glycoprotein showed that it contained less sialic acid than that of normals [3].

IαI Related Proteins in Stones

Regardless of mineral composition, all urinary stones contain an organic material refereed to as matrix protein [35, 36]. The latter represents about 2-5% by

weight and consists predominantly of proteins [12]. Its role in the formation of urinary stones remains a subject of research. To better understand this role, many investigators attempted to analyze proteins extracted from urinary stones. Unfortunately, few works were devoted to a search for IαI and its related peptides and to quantify them. Petersen *et al.* [48] have found hemoglobin and two serine proteases in the extract of CaOx kidney stone. A protease inhibitor, α1-antitrypsin, has been extracted and quantified suggesting that some stones are in contact with blood during their development [70]. Hoyer [31] has extracted several proteins from CaOx monohydrate stones and quantified them. His results showed that uropontin, or osteopontin, constituted the major protein in the extract (90 μg/100 mg). Tamm-Horsfall protein, albumin, and IαI fragment represent less than 10 μg/100 mg of stones. Recently, in our laboratory we identified proteins extracted from CaOx crystals produced *in vitro* in human urine by adding sodium oxalate [5]. Among the proteins, we detected a fragment of IαI, which could be bikunin, but in lesser amounts compared to prothrombin F1 and osteopontin. More experiments are in progress.

Concluding Remarks

It seems that UAP is biochemically similar to bikunin, the light chain of IαI. But functionally these two proteins appear different. IαI becomes a family of proteins involved in modulating calcium oxalate crystallization and stone formation. Another plasma protein, the F1 fragment of prothrombin has also been implicated in calcium oxalate stone formation [64]. Apparently, the crystallization of calcium oxalate is modulated by a variety of macromolecules including many plasma proteins.

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Discussion with Reviewers

B. Hess: How do the authors explain the suggested reabsorption of IαI in the proximal tubule, i.e., how does a protein with a molecular weight of 180-220 kDa get across the tubular membrane into the tubular fluid?
Reviewer VII: The question of whether bikunin/UAP (or IαI) is synthesized in the kidney, or is merely filtered at the glomerulus is not directly addressed. Do the authors have any data to define more precisely the nature of the IαI reactivity?
Authors: IαI is mainly synthesized in the liver and secreted in plasma. Recent work studying the expression of mRNA coding for IαI confirmed its exclusive synthesis in the liver [55]. The presence of a positive IαI immunoreactivity in kidney is due to the presence of bikunin which has a molecular weight in a range of 35-45 kDa, and can easily pass through the glomerular filters, thus, it can be recognized by IαI polyclonal antibody.

B. Hess: Similarity of nephrocalcin (NC) and HI-14 of IαI/bikunin: nephrocalcin contains Gla; what is the Gla-content of HI-14 of IαI/bikunin?
Authors: Nephrocalcin is known to have 2-3 Gla residues. However, HI-14 or any protein related to IαI does not have any Gla residue. Interestingly, in a recent work, Tang *et al.* [66] reported that NC is HI-14 by using similar techniques described by Nakagawa to isolate NC. The authors suggested that NC was contaminated by other proteins containing Gla, like prothrombin F1, to explain the presence of these residues in the preparation of NC. Nonetheless, another feature of NC is it is synthesized in kidney, but HI-14 is not. Therefore, similarity or identity of NC to HI-14 remains speculative and needs confirmation by, e.g., using NC antibody.

B. Hess: It should be acknowledged that there are certain requirements for an inhibitor/modifier to be of clinical significance for calcium stone formation, such as: (1) abundance in urine, (2) occurrence of calcium binding sites in the molecule, (3) structural and functional abnormalities or significantly reduced excretion in active stone formers versus healthy people, and (4) possibly some therapeutic implications, i.e., altering physico-chemical conditions of urines, modifying the diet or prescribing certain drugs should improve the functional status of a given macromolecule towards more inhibition of crystallization, mainly crystal aggregation.
Authors: In this paper, we do not attempt to describe UAP as a superinhibitor to be considered and the others to be discarded. In the paper where UAP was described for the first time [2], we pointed out that one inhibitor alone cannot be responsible for the total urinary inhibitory activity. Moreover, in a second paper [3], we reported that UAP extracted from the urine of stone forming subjects was less inhibitory compared to that isolated from the urine of healthy persons. We suggested that this diminution does not account for the total urinary inhibitory activity, but that other protein inhibitors also may be defective.

M. Daudon: The authors suggest that variation in urinary excretion of IαI related proteins may occur in various pathological conditions responsible for an increased excretion of bikunin. Do the authors have some information about the normal range of UAP and bikunin excretion in human urine?
Authors: The normal range of urinary excretion of IαI related proteins is about 2 to 10 mg/day according to the method used [77, 78]. This excretion can be enhanced to 50-100 fold or even more in certain pathological conditions such as cancer [75, 76].

M. Daudon: How can the authors explain the weaker inhibitory activity of UAP reported in this paper as compared to that reported in a previous paper [4]?
Authors: The weaker inhibitory activity of UAP in this study is not significant compared to that reported in the

previous paper [4]. Several factors can contribute to this diminution. The two studies were done in different laboratories (France versus USA). The factors may include the reagents, materials, and urine samples.

Y. Nakagawa: The inhibition assay was the ^{45}Ca deletion method. Any negatively charged molecules, including chondroitin sulfate, and dermatan sulfate, will bind to calcium; those compounds would show inhibitory activity by this method. IαI binds Zn^{2+}, and so it is not surprising that it would bind to Ca^{2+}, and show apparent crystal growth inhibition.

Authors: Albumin was tested in this model and showed no effect on crystallization (unpublished data). Tamm-Horsfall protein was also tested and showed only a minor effect [2]. Moreover, by using the same assay, we were able to differentiate between UAP isolated from the urine of normal subjects and that isolated from stone forming subjects [3]. The reviewer considers that it is not surprising that IαI shows apparent crystal growth inhibition since it binds to divalent cations such as Ca^{2+} and Zn^{2+}. However, we believe that as far as urolithiasis is concerned, whatever the mechanism involved, the important fact is the inhibition of CaOx crystal formation.

J.P. Salier: In reference [4], the authors indicate that chondroitinase or hyaluronidase can split UAP into two 20 kDa polypeptide chains that would be attached by GAG bond in the intact UAP molecule. Is this conclusion strengthened by the isolation of both 20 kDa chains? Are they somehow different from each other? Alternatively, is the authors' conclusion based upon the electrophoretic behavior of the enzyme-treated UAP that provides a 20 kDa band? Is there any chance that UAP is made up of one 20 kDa polypeptide and a hyaluronidase-sensitive GAG structure, as bikunin is?

Authors: In a previous paper [4], preliminary molecular characteristics of UAP were reported. Among them, we treated UAP with chondroitinase and then subjected to polyacrylamide gel electrophoresis analysis and amino acid sequencing. The results obtained from the polyacrylamide gel showed a band with a molecular weight of 20 kDa. However, the amino acid sequence determined in a protein laboratory showed two identical sequences. Therefore, we suggested that UAP is made of two polypeptide chains attached via a chondroitin chain. However, in a recent paper (Atmani *et al.*, Eur. J. Bioche., 1996, in press), we repeated the same experiment and we demonstrated that treatment of UAP with chondroitinase yielded only one peptide chain at 20 kDa, confirmed by a unique amino acid sequence. This new data is in agreement with those reported previously for bikunin by other investigators [8, 61]. Based on this new data, we concluded that UAP is bikunin.

J.P. Salier: The central issue of whether or not bikunin, UAP, and the protein published by Sørensen *et al.* [63] are identical or at least originate from a same gene remains unanswered. Could the authors provide a tentative conclusion?

Authors: It is generally agreed that proteins may be affected by technical procedures used in different laboratories for their purification, including the reagents and the conditions of experiments. A good example is Tamm-Horsfall protein which has been demonstrated to be a promoter, inhibitor, and inactive toward CaOx crystal formation. Therefore, it is not surprising that bikunin, UAP, and Sørensen's protein, isolated in different laboratories, are in fact identical or at least derived from the same gene. Thus, it is important to standardize the technique used for the purification of proteins as well as the assays of CaOx crystallization.

J.P. Salier: The authors should outline that mRNA detection is likely to be more precise than antibody-assisted detection. The specificity, cross-reactivity, and associated background of some antibodies has not always been considered conclusively.

Authors: We agree with the reviewer that mRNA detection is more specific than antibody detection. Here, we wanted to point out that even if IαI is synthesized in liver, some of the proteins related to it are present in different tissues. Their presence may have an important role such as the inhibition of CaOx crystal formation in the kidney.

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