

7-25-1993

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Deganello, Sergio (1993) "The Interaction Between Nephrocalcin and Tamm-Horsfall Proteins with Calcium Oxalate Dihydrate," *Scanning Microscopy*. Vol. 7 : No. 3 , Article 34.

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THE INTERACTION BETWEEN NEPHROCALCIN AND TAMM-HORSFALL PROTEINS WITH CALCIUM OXALATE DIHYDRATE

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(Received for publication January 28, 1993, and in revised form July 25, 1993)

Abstract

Studies of crystals of calcium oxalate dihydrate (COD) grown by vapor diffusion from solutions containing 5.1×10^{-7} , 1.5×10^{-6} , and 1.0×10^{-5} M nephrocalcin (NC), indicate that NC profoundly affects COD's habit, size and structure. The decrease in COD size is such that at 1.0×10^{-5} M NC, the dimensions of the crystals are reduced about five-fold with respect to those of a NC-free control. In addition, the planes of the {101} form disappear, the original habit is lost, and the diffraction pattern deteriorates to such an extent that only the 200 reflections are recorded.

The results are quite different when NC is adsorbed upon rigid substrates. Under such conditions, NC acts as a promoter and not as an inhibitor of growth and thus nucleates COD from its {100} planes. Consequently, COD grows systematically juxtaposed on NC. This effect is highly reproducible and stereospecific.

COD crystals grown by vapor diffusion from solutions exposed to increasing concentrations of Tamm-Horsfall protein (THP) exhibit a drastic decrease in COD's self-association. In sharp contrast with the results obtained for NC, precession photographs taken of COD samples exposed to 1.2×10^{-5} M THP do not show evidence of deterioration of COD diffraction patterns with respect to a protein-free control. Furthermore, THP neither nucleates COD, nor does it appear to influence its growth or habit even when THP is immobilized upon a rigid substrate.

Key Words: Calcium oxalate dihydrate, nephrocalcin, Tamm-Horsfall protein, growth inhibition, structural interactions, oxalate stones.

Introduction

We have reported elsewhere on the interaction between human nephrocalcin (NC, an acidic glycoprotein found in mammals and humans; Nakagawa *et al.*, 1983; 1987), and calcium oxalate monohydrate (COM; Deganello, 1991). Those findings provided a basis for a quantitative understanding of the structural requirements necessary to promote or inhibit the growth of COM. Here, we study the effect of NC and Tamm-Horsfall glycoprotein (THP) on calcium oxalate dihydrate (COD). Intuitively, it would be expected that NC would interact with COD in a manner similar to the way it does with COM, due to the structural similarities that exist between the two oxalates (Deganello, 1986). On the other hand, no anticipation is possible with regard to the role of THP on the nucleation and growth of COD since it is not even clear whether THP interacts with COM or is altogether involved in kidney or bladder stone formation (e.g., Kitamura and Pak, 1982; Scurr and Robertson, 1986; Worchester *et al.*, 1988). To the best of our knowledge, nothing is known about the interaction between THP and COD at the atomic-molecular level. Furthermore, THP is remarkably different from NC, although NC is rich in acidic residues (e.g., Tamm and Horsfall, 1950; Kumar and Muchmore, 1990; etc.). It would thus appear that any information regarding the interactions between COD, THP and NC is likely to be of considerable value in understanding the role that different classes of glycoproteins may have on the nucleation and growth of the calcium oxalates.

The experimental data presented here were gathered from crystals grown by vapor diffusion (Deganello, 1992) and interpreted in terms of variations in size, habit and crystal structure as determined by X-ray diffraction (powder-single crystal) and scanning electron microscopy.

Materials and Methods

Nephrocalcin and Tamm-Horsfall protein were extracted and purified from human urine by Dr. Yasushi Nakagawa (Univ. Chicago) who made them available to us (Nakagawa *et al.*, 1983; Hess *et al.*, 1991). Crystals

of COD were grown at room temperature by diffusing vapors of oxalic acid into aqueous solutions of calcium chloride. Two sets of experiments were carried out: one was intended to ascertain whether aqueous solutions containing NC and/or THP inhibited the growth of COD, while the other tested the ability of NC and THP to induce the growth of COD once they were immobilized upon a rigid substrate. Since the growth protocols for COD have been reported elsewhere (Deganello, 1992), we provide here just enough information to clarify the ensuing discussion.

To study the interaction between COD and NC when this is dispersed in solution, 1 ml CaCl_2 (0.3 M) and 300 μl Tris-THAM (0.5 M; pH 7.4) were pipetted into a glass cylinder (diameter 18 mm). This cylinder was introduced into the B3 well (diameter 18 mm) of a Falcon culture plate (nr. 3047; 24 wells) and used as a control. One ml CaCl_2 (0.3 M) and 300 μl Tris-THAM (0.5 M; pH 7.4) were also introduced into three additional glass cylinders in B4, C3, and C4 together with varying aliquots of NC. The final concentrations of NC were 5.1×10^{-7} , 1.5×10^{-6} , and 1.0×10^{-5} M, respectively. Furthermore 2 ml Tris-THAM (pH 7.3; 0.5 M) was pipetted into A2, B2, C2, D2, A3, D3, A4, D4, A5, B5, C5 and D5. Then 1 ml H_2O and 20 μl diethyloxalate (Baker, H888-07, Phillipsburg, NJ) were introduced into A1, B1, C1, D1, A6, B6, C6, and D6. Finally, the plate was closed and its cover sealed in place with three layers of electrical tape in order to prevent vapor loss. Water hydrolyzes diethyloxalate to oxalic acid (Elving and Chao, 1949); consequently 20 μl diethyloxalate was added daily with a Hamilton syringe into A1, B1, C1, D1, A6, B6, C6, and D6 (total diethyloxalate: 100 μl) in order to gradually increase the concentration of oxalic acid. Since the Falcon plate was closed, the syringe was inserted into the latter through eight orifices (~ 0.6 mm diameter) that were predrilled in the cover over the wells A1, B1, C1, D1, A6, B6, C6, and D6. No significant vapor loss was incurred during such procedure since the orifices were shielded from the outside by a 0.2 mm-thick rubber gasket. About 72 hours after the plate was closed, enough oxalic acid had diffused into the calcium chloride solutions contained in B3, B4, C3 and C4 to induce nucleation of COD. Typically, these COD crystallites were allowed to grow for a total of 168 hours prior to washing in distilled water, rinsing in absolute ethyl alcohol, and drying under dry nitrogen.

COD was then grown in the presence of THP. The growth criteria used were identical to those reported earlier with the exception that six protein concentrations were tested. Consequently, two culture plates were needed for each set of experiments. In one, the concentration of THP was 2.6×10^{-7} , 5.2×10^{-7} and 1.2×10^{-6} M (B4, C3 and C4); in the other, THP was 2.6×10^{-6} , 5.2×10^{-6} and 1.2×10^{-5} M. B3 was the protein-free control in both plates.

Finally, all of the above studies were repeated under the identical experimental conditions without using the glass cylinders. To do so, the solutions were intro-

duced directly into the plastic wells of the culture plates in order to determine whether their surfaces influenced the process of crystallization.

In order to test the ability of NC and THP to nucleate COD, each of the two proteins was allowed to adsorb upon the bottom of B4, C3 and C4 in a Primaria plate (Falcon, nr. 3087). This plate, although similar to model nr. 3047, is treated with amide and amino-functional groups which make its surface positively charged with the result that proteins readily adhere to it. To adsorb NC, two culture plates were used. In one, 1 ml CaCl_2 (0.3 M), 0.3 ml Tris-THAM (0.5 M; pH 7.4) and 1.5×10^{-7} , 3.0×10^{-6} and 8×10^{-6} M NC were introduced in B4, C3 and C4. In the other one, NC was 1.5×10^{-6} , 3.0×10^{-5} and 8×10^{-6} M. As usual, B3 was the protein-free control in both plates [1 ml CaCl_2 (0.3 M) with 0.3 ml Tris-THAM (0.5 M; pH 7.4)]. THP was immobilized upon B4, C3 and C4 using the same methods adopted for NC. However, since the results that were obtained for the two proteins, although highly self-consistent, were drastically different, we also adsorbed THP upon B4, C3 and C4 according to a different procedure. Using a Costar plate nr. 3524, 0.4×10^{-4} , 0.4×10^{-5} and 0.4×10^{-6} M THP solutions (pH 7.4) were allowed to adsorb upon B4, C3 and C4 for 12 hours. The solutions were aspirated out of the wells, these were rinsed with 2 ml CaCl_2 (0.3 M) and 1 ml CaCl_2 (0.3 M), together with 30 μl Tris-THAM (0.5 M; pH 7.4), was introduced into B3, B4, C3 and C4. Two ml of Tris-THAM (0.5 M; pH 7.4) was pipetted into A2, B2, C2, D2, A3, D3, A4, D4, A5, B5, C5 and D5 while 20 μl diethyloxalate and 1 ml H_2O were introduced into A1, B1, C1, D1, A6, B6, C6, and D6. Finally, the culture plate was closed and crystallization proceeded as usual by programming the concentration of diethyloxalate in A1, B1, C1, D1, A6, B6, C6, and D6 (20 μl / day for a total of 100 μl).

A Costar plate (nr. 3596) consisting of 96 wells having approximately one tenth the capacity of those of model nr. 3524 was used. Consequently, the volumes of solution used were reduced accordingly while 72 of the 92 wells were occluded with electrical tape in order to retain the growth conditions previously reported.

Double distilled water was used throughout. Routinely, all the experiments were carried out in triplicate in order to control their reproducibility. In no case was there any apparent indication that either NC or THP denatured, in agreement with our observations regarding the extreme reproducibility of the results.

Following optical observations (plane and polarized light), representative material from each growth batch was studied by scanning electron microscopy (SEM) and powder and single-crystal X-ray diffraction. The SEM analysis was carried out with an ETEC microscope operated at 25 kV using samples mounted on aluminum stubs with a silver-based cement and treated with gold evaporated by glow discharge under vacuum. The powder diffraction patterns were obtained from single crystals (Gandolfi camera; 114.6 mm; 38 kV, 18 mA,

Ni-filtered Cu K α ; 3×10^{-3} torr) as well as powders (Guinier camera; quartz-monochromated CuK α_1). Finally the single-crystal work was carried out by zero-level precession photography (Zr-filtered MoK α ; 40 kV, 20 mA) and four-circle diffractometry. For the former, the study included samples exposed to, 1.5×10^{-6} and 1.0×10^{-5} NC as well as 1.2×10^{-5} M THP. For the latter, 2200 intensities were collected on a SYNTEX P2₁ automated diffractometer (graphite-monochromated MoK α radiation) using a specimen ($\sim 0.08 \times 0.07 \times 0.06$ mm) which grew upon C4 when this was adsorbed with 8×10^{-6} NC. Data reduction and analysis were carried out using the package assembled by Sheldrick (1976) and applying the atomic coordinates by Tazzoli and Domeneghetti (1980).

Results

The adsorption of nephrocalcin, even at the lowest concentration tested, inhibits the growth of calcium oxalate dihydrate both in the plastic wells as well as in the glass cylinders. This decrease in size manifests itself (Figs. 1a, 1d) to such an extent that at 1.0×10^{-5} M NC the dimensions of the samples are reduced about 5-fold with respect to those growing in the NC-free control. In addition, the planes of the {101} form disappear, the original habit is lost and a few crystallites assume a dumb-bell shape [previously produced by us when COM was exposed to 200×10^{-7} M NC (see Figure 1f in Deganello, 1991)]. This debilitating effect on the part of NC is corroborated by zero-level precession photographs, [001] projections, taken of single crystals exposed to, 1.5×10^{-6} and 1.5×10^{-5} M NC. At 1.5×10^{-6} M NC, the diffraction pattern of COD shows evidences of twinning, streaking and loss of long-range order. At 1.5×10^{-5} M NC, such disturbances worsen to the point that disorder is extreme and only the 200 reflections and their symmetry-equivalents remain in the diffraction pattern (Figs. 2a, 2b). Interestingly, these are the very reflections that monitor the behavior of the {100}, since the 100's are systematically extinct in COD (space group, S.G., I4/m).

No such results are obtained when NC is adsorbed upon a rigid substrate. Under these conditions, NC acts as a promoter, rather than as an inhibitor, of crystal growth. Specifically, NC nucleates COD from the {100} with the result that the latter grows systematically juxtaposed on NC (Fig. 3). The effect, which is best observed in the wells with higher protein concentration (C4 and C3), is highly reproducible and specific, in agreement with the results of the three-dimensional refinement earlier discussed. This refinement converged to a value $R = 0.05$ of the conventional crystallographic discrepancy index and evidenced the presence of a number of spurious peaks (range: $1-3 \text{ e}\text{\AA}^3$) in the (100) plane. No other indication of disordering was detected in the rest of the structure. Furthermore, all the atomic positional parameters were found to be identical, within the value of the standard deviation, to those obtained by Tazzoli and Domeneghetti (Table 1).

The results of the interaction of THP with COD are considerably different. As the concentration of this protein increases so does its tendency to gel; concomitantly the self-aggregation of COD drastically decreases (Figs. 4a, 4c). This decrease is associated with an increase in crystal size. At 1.2×10^{-5} M THP, the self-aggregation of COD is reduced to such a level that it is difficult to assess whether it could be further affected by any other increase in protein concentration. However, the crystals of COD that grow in proximity of the bottom of the wells (cylinders) show a characteristic increase in the surface area of their {100}'s (Fig. 5). No such habit modification is detected in the crystals growing at or by the surface of the solutions nor is it ever observed in any crystal, regardless of its site of growth, if THP is less than 2.6×10^{-6} M. In addition, in no case does the protein inhibit the growth of COD. Furthermore, contrary to the results obtained with NC, precession photographs taken of 10 samples exposed to 1.2×10^{-5} M THP do not show any evidence of deterioration of their diffraction pattern [hk0 and 0kl projections] with respect to a protein-free control (Fig. 6). Finally, never does THP nucleate COD, nor appear to influence its growth or habit when it is immobilized upon B4, C3 and C4.

With all of these interactions, there were no significant changes in the values of the lattice parameters of COD whether this interacted with NC or THP. Those parameters remained $a = 12.35$, $c = 7.364(2)$ Å for I4/m. This conclusion is clearly supported by the results of the X-ray powder diffraction data (Table 2).

Discussion

When it is free to interact in solution with COD, NC shields the planes of the {100} form and, to a lesser degree, those of the {101} from the rest of the solution (Figs. 1a, 1c). Thus, the growth of COD is inhibited in directions perpendicular to all of those faces which produces an overall decrease in crystal size as the concentration of NC increases. Such a process of protein adsorption is not likely to be controlled by specialized Ca-Ca interactions. Although one cannot exclude *a priori* that they may have some importance, the bulk of the process of protein adsorption, and thus crystal-growth inhibition, is controlled by the highly selective bonding interaction that NC establishes with the {100}. These are the faces which offer maximum resistance to structural destabilization; as such they provide both the Ca-centered polyhedral chains as well as the oxalate-water-oxalate ribbons that link those chains to one another and, thus, make three-dimensional growth possible. This condition of minimum binding energy is stipulated through a set of oxalate ions that are normal to the highly planar arrangement of the {100}'s and, thus, are ideally suited to bind with NC (Fig. 7), just as was the case for their C(3) C(4) counterparts in COM (Deganello, 1991). Such a specialized organization is unique to the {100} in COD as was to the $(\bar{1}01)$'s in COM.

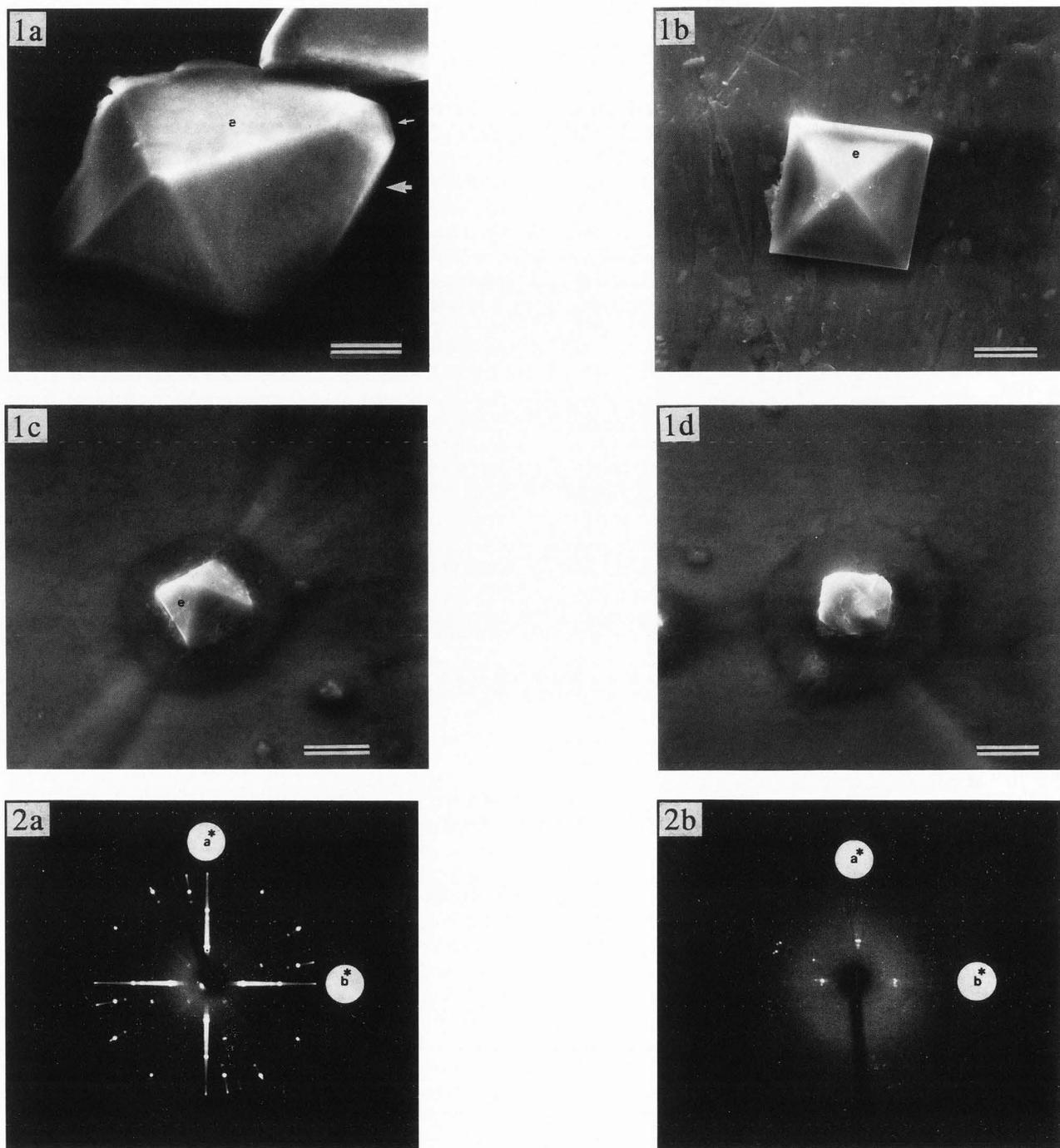


Figure 1. (a) Sample of COD grown in absence of NC. Notice the well developed, apical e plane of the $\{101\}$ form and its symmetry equivalents. Larger arrow indicates c plane belonging to $\{100\}$. Smaller arrow points to plane belonging to $\{011\}$. (b) Sample of COD grown in presence of 5.1×10^{-7} M NC. Notice overall decrease in crystal size. (c) Sample of COD grown in 1.5×10^{-6} M NC. Size has reduced even further. (d) Sample grown in 1.0×10^{-5} M NC. The e planes of the $\{101\}$ form are no longer visible. Notice crystal deterioration. Bars = 10 μm .

Figure 2. (a) $hk0$ precession photograph of a COD control grown in absence of NC. Unfiltered $\text{MoK}\alpha$; 38 kV, 18 mA, $\mu = 10^\circ$, 40 minutes, 3000 ASA film; S.G. I4/m. (b) $hk0$ precession photograph of a COD sample grown in presence of 1.0×10^{-5} M NC. Notice splitting of 200 reflections. Only these reflections and their symmetry equivalents are recorded after a 5 hour exposure time. Unfiltered $\text{MoK}\alpha$; 38 kV, 18 mA, $\mu = 10^\circ$, 5 hours, 3000 ASA film.

Atomic Basis of Inhibition of COD

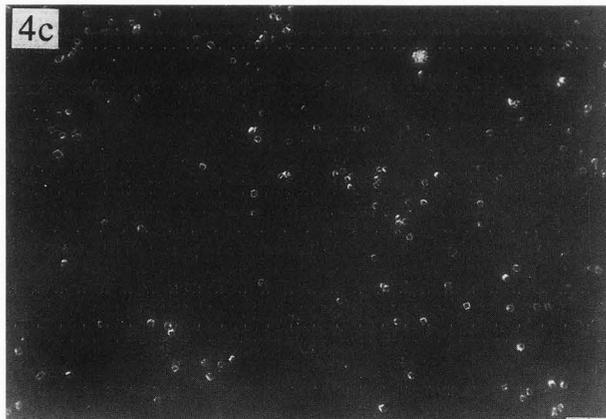
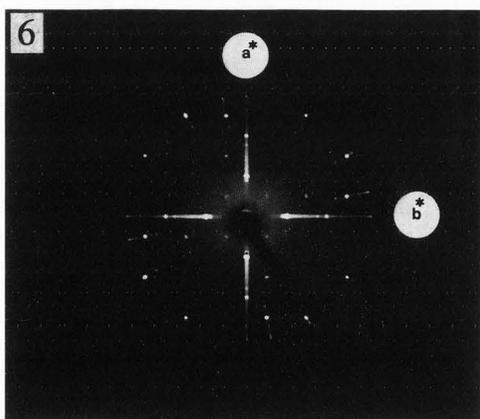
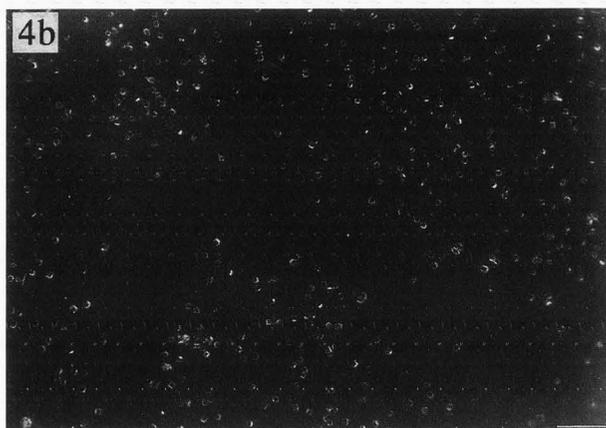
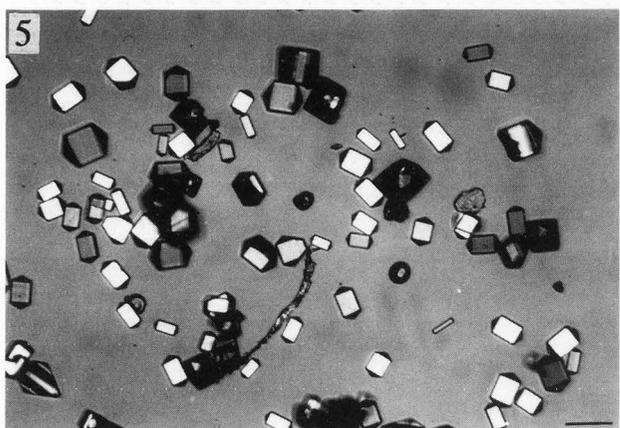
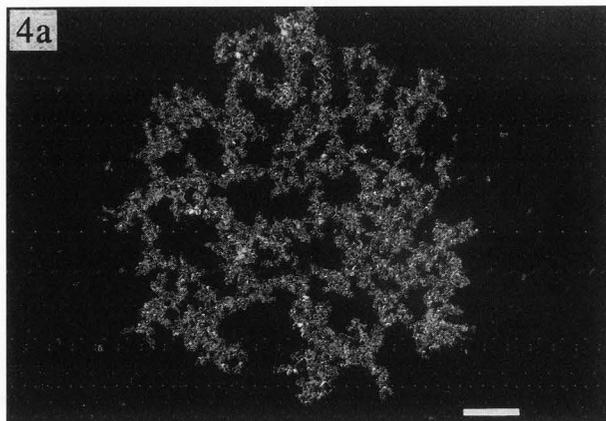
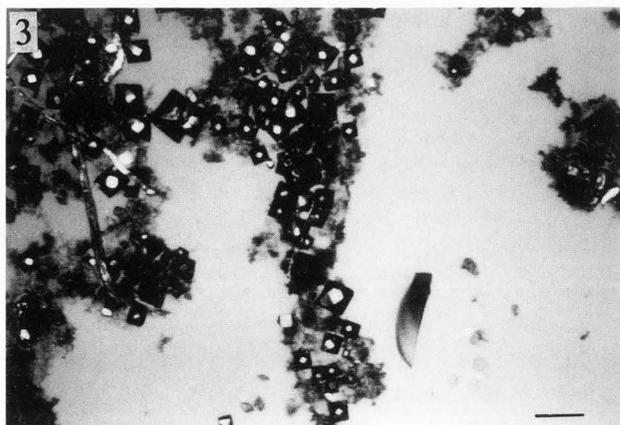


Figure 3. Optical micrograph of COD crystals nucleated by 8×10^{-6} M NC when this is adsorbed upon C4. Notice COD grows juxtaposed on NC using the (100)'s as the common interface. Bar = 100 μ m.

Figure 4. Optical micrographs at identical magnifications of nucleation of COD crystals: (a) in 5.1×10^{-7} M NC (notice self-aggregation of COD); (b) in 5.2×10^{-7} M THP (notice drastic decrease in self-aggregation of COD); and (c) in 1.2×10^{-5} M THP [self-aggregation of COD has decreased even further with respect to (b)]. Bar = 100 μ m.

Figure 5. Optical micrograph of COD crystals growing on the bottom of C(4) when THP is 1.2×10^{-5} M. Notice characteristic increase in surface area of the (100)'s. Bar = 100 μ m.

Figure 6. hk0 precession photograph of a sample of COD exposed to 1.2×10^{-5} M THP. There is no evidence of deterioration of the diffraction patterns with respect to the control (Fig. 2a). Unfiltered MoK α ; 38 kV, 18 mA, $\mu = 10^\circ$, 2 hours, 3000 ASA film.

Table 1. Selected atomic coordinates of a COD sample nucleated by 8×10^{-6} M NC.

	This work			Tazzoli and Domeneghetti (1980)		
	x/a	y/b	z/c	x/a	y/b	z/c
Ca	.1996(2)	.3029(2)	.0	.1993(1)	.3011(1)	.0
C	.4461(3)	.2420(3)	.1055(3)	.4464(2)	.2415(2)	.1053(3)
O(1)	.3562(3)	.2460(4)	.1831(3)	.3564(1)	.2458(1)	.1828(1)
O(2)	.2358(4)	.4638(4)	.1802(4)	.2355(2)	.4634(1)	.1799(2)
W(1)	.1487(5)	.1148(5)	.0	.1490(3)	.1145(2)	.0
W(2)	.0188(5)	.3844(6)	.0	.0192(2)	.3841(3)	.0

Ca stands for the calcium atom, C for carbon atom, while O(1) and O(2) refer to the oxygen atoms. W(1) and W(2) stand for the oxygens of the two water molecules.

Table 2. Selected powder diffraction data of COD crystals grown in presence of NC and Tamm Horsfall protein.

S1	S2	S3	ASTM		
d(Å)	d(Å)	d(Å)	d(Å)	I/I ₁	hkl
8.7	8.7	8.7	8.73	4	110
6.2	6.2	6.2	6.18	100	200
4.4	4.4	4.4	4.42	30	211
3.68	3.68	3.68	3.68	12	002
3.09	3.09	3.09	3.09	10	400
2.81	2.81	2.81	2.815	14	222
2.77	2.77	2.77	2.775	65	411
2.404(2)	2.403(2)	2.402(2)	2.408	16	103
2.241(2)	2.241(2)	2.242(2)	2.243	25	213
1.956(2)	1.957(2)	1.956(2)	1.957	10	611

S1 and S2 refer, respectively, to crystals of COD grown in 0 and 1.5×10^{-5} M NC. S3 refers to crystals of COD grown in 1.2×10^{-5} M THP. ASTM refers to file nr. 17-541 of the Powder Diffraction File of the American Society for Testing Materials.

When NC is 1.5×10^{-6} , neither the {100} nor the {101} show a drastic decrease in their growth rate relative to one other, indicating that both sets of faces are equivalently shielded by NC. It would, therefore, appear that the higher adsorption efficiency of the {100} is mitigated by charge-density effects that the polyelectrolyte-like nature of NC induces over the {101}. Such effects are not likely to be negligible, since the planes of the {101} occupy over 90% of the available surface area. However, at 1.5×10^{-5} M NC, the {101} disappears, but the {100} remains and so do the 200 reflections (Fig. 2). Such a specificity of NC for the {200}'s and, thus the {100}'s, becomes particularly evident when the protein is adsorbed upon a rigid substrate. Under

such conditions, NC almost exclusively nucleates COD from the {100} (Fig. 3) and, in the process, stabilizes those nuclei since it lowers their energy of nucleation. Consequently their growth is favored and can continue undisturbed since NC remains confined to the substrate. The resulting NC-nucleated crystals are therefore expected to be morphologically well developed and characterized by a considerable degree of structural order, with the exception of the {100} since these planes host the atomic interface of COD with NC. All of these inferences are confirmed by the growth experiments which produced excellent crystals measuring up to 200-250 μm along [100], as well as by the results of the three-dimensional refinement.

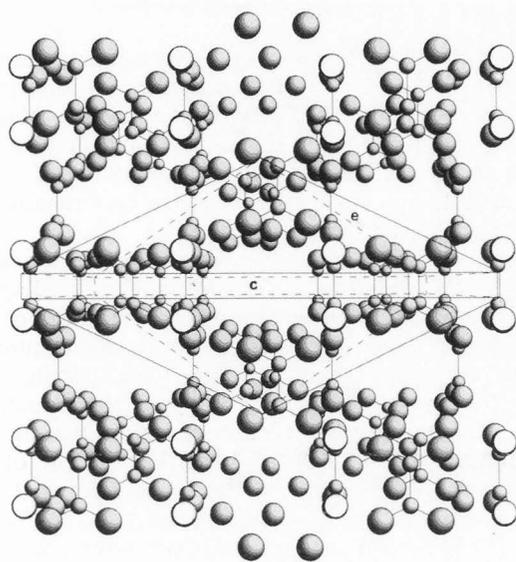


Figure 7. A schematic view of the COD structure. For the sake of convenience, the oxalate groups that are perpendicular to the highly planar surface of the {100}, here identified as *c*, are marked in white. Notice that only those groups are available for bonding to NC.

In light of all of this, the process of NC adsorption may be thought to take place in two stages. First, NC adsorbs upon the {100} which results in local disorder since it disturbs the oxalate groups that link the Ca-centered polyhedral chains to one another along *b*. Second, such an interface serves as a site of growth for COD as long as the side chains are confined within it. Otherwise, if the chains of NC emerge from it and interact with the growing nuclei of COD, as is the case when the protein is dispersed in the calcium chloride solutions, they interfere with the oxalate-water-oxalate ribbons that link the calcium polyhedra along *c*. Consequently, the coordination of those polyhedra is eventually impaired and so is the effectiveness of the hydrogen bond network with the result that three-dimensional growth is disturbed. The expression of these phenomena is concentration dependent. Nephrocalcin concentrations of 1.5×10^{-6} M are already adequate to destabilize the structure and, thus, negatively affect crystal size. At 1.5×10^{-5} M, such a destabilization is drastic with the result that size is reduced even further since the structure is much less efficient at organizing itself. Such a dependence on protein-concentration is also reflected in the kinetics of the process of growth. During crystallization, the first crystallites of COD were observed to form about 90 hours after the culture plate was closed (1.5×10^{-5} M NC; C4). However in C3, where the concentration of NC was 1.5×10^{-6} M, the onset of nucleation was detected just after 62 hours. Such a capacity

on the part of NC of modulating the processes of growth inhibition and nucleation through the same atomic interface is striking. This property is likely to be shared by other proteins and should have general applicability to other classes of interaction, such as those reported by Campbell *et al.* (1989) with regard to the dual role of selected polyelectrolytes and proteins. These, in fact, behave both as promoters as well as inhibitors of COM crystallization.

The results of the interaction between THP and COD were quite different. While NC favors COD aggregation, THP militates against it, leading to the conclusion that the presence of the protein causes increased repulsion at the crystal/solution interface. The magnitude of this effect increases with increasing protein concentration and is particularly evident in calcium solutions since they favor the natural propensity of THP to gel (Kumar and Muchmore, 1990). Because of this, oxalic acid cannot effectively diffuse inside the solutions in B4, C3 and C4 and, thus, the value of the Ca/oxalate ratio is considerably lower at the surface than at the bottom of those solutions. This high value of the Ca/oxalate ratio favors the development of the {100}, even in absence of THP (Deganello, 1992). However, under such conditions, those planes do not develop to the degree that was observed in this work, suggesting that THP contributes to their development by shielding them from the crystallizing solution. However, even if this were the case, such interaction is of limited scope since it never results in structural destabilization nor affects crystal size nor promotes the growth of COD, irrespectively of the amount of THP (up to 0.4×10^{-4} M) that is adsorbed upon B4, C3 and C4. One therefore wonders about the mutual role of THP and NC during crystallization. Since the two proteins have such a profoundly different effect on the processes of growth promotion and inhibition, the question arises as to whether or not their concomitant presence may indeed be a necessary prerequisite to modulate crystal growth. This is so for the combined action of NC and THP would necessarily prevent the development of large crystals and favor, instead, the formation of those that are small and have relatively uniform sizes.

Finally, the insensitivity to changes that was exhibited by the lattice parameters of COD when it was exposed to NC or THP, indicates that the process of protein adsorption is quite contained as it should be, due to the limited void availability of the COD structure type.

Acknowledgments

Grant DK-33949 from the National Institutes of Health in the early stage of the work is acknowledged. Continued support was provided by the Italian CNR. Dr. Y. Nakagawa was instrumental in providing both the nephrocalcin as well as the Tamm-Horsfall protein used in this study.

References

Campbell AA, Ebrahimpour A, Perez L, Smesko ASA, Nancollas GH (1989). The dual role of polyelectrolytes and proteins as mineralization promoters and inhibitors of calcium oxalate monohydrate. *Calcif. Tissue. Int.* **45**: 122-128.

Deganello S (1986). Phase transitions of calcium oxalate trihydrate and epitaxy in the weddellite-whewellite system. *Scanning Electron Microsc.* **1986**;IV: 1721--1728.

Deganello S (1991). Interaction between nephrocalcin and calcium oxalate monohydrate: a structural study. *Calcif. Tissue. Int.* **48**: 421-428.

Deganello S (1992). Growth by vapor diffusion of calcium oxalate dihydrate, calcium oxalate trihydrate and calcite. *Science and Technology for Cultural Heritage* **1**: 1-8.

Elving PJ, Chao PC (1949). Determination of alkali metals in silicates and similar materials. *Anal. Chem.* **21**, 507-510.

Hess B, Nakagawa Y, Parks JH, Coe FL (1991). Molecular abnormality of Tamm-Horsfall glycoprotein in calcium oxalate nephrolithiasis. *Am. J. Physiol.* **260**, F569-F578.

Kitamura T, Pak C (1982). Tamm and Horsfall glycoprotein does not promote spontaneous precipitation and crystal growth of calcium oxalate *in vitro*. *J. Urol.* **127**: 1024-1026.

Kumar S, Muchmore A (1990). Tamm-Horsfall protein-uromodulin (1950-1990). *Kidney Int.* **37**: 1395-1401.

Nakagawa Y, Abram V, Kedzy FJ, Kaiser ET, Coe FL (1983). Purification and characterization of the principle inhibitor of calcium oxalate monohydrate crystal growth in human urine. *J. Biol. Chem.* **20**: 12594-12600.

Nakagawa Y, Deganello S, Chou C, Ahmed M, Coe FL (1987). Isolation from human calcium oxalate renal stones of nephrocalcin, a glycoprotein inhibitor of calcium oxalate crystal growth. *J. Clin. Invest.* **79**: 1782-1787.

Scurr DS, Robertson WG (1986). Modifiers of calcium oxalate crystallization found in urine. III. Studies on the role of Tamm-Horsfall mucoprotein and of ionic strength. *J. Urol.* **136**: 505-507.

Sheldrick GM (1976). SHELX. Program for structure determination. University of Cambridge, England.

Tamm I, Horsfall FL (1950). Characterization and separation of an inhibitor of viral hemagglutination present in urine. *Proc. Soc. Exp. Biol. Med.* **74**: 108-114.

Tazzoli V, Domeneghetti C (1980). The crystal structures of whewellite and weddellite: re-examination and comparison. *Amer. Min.* **65**: 327-334.

Worcester E, Nakagawa Y, Wabner CL, Kumar S, Coe FL (1988). Crystal adsorption and growth slowing by nephrocalcin, albumin and Tamm-Horsfall protein. *Am. J. Physiol.* **255**: F1197-F1205.

Discussion with Reviewers

A.A. Campbell: Did you quantify the amount of protein adsorbed on the COD crystals by performing adsorption isotherm experiments? What is the calcium binding affinity for the proteins investigated and how is this important to adsorption and/or promotion of COD growth? Do you have any idea of the conformation of the immobilized protein molecules?

Author: The calcium binding affinity for nephrocalcin is 1.53×10^{-7} M. However data on calcium binding affinity are not necessarily particularly indicative of the efficiency of the processes of adsorption and/or promotion of growth whenever those are stereospecific. No adsorption isotherm experiments were carried out for COD; however, the overall indications are that the adsorption of NC and THP upon COD should be of the same order of magnitude of that measured on COM (Worcester *et al.*, 1988). Unfortunately the conformation of the immobilized proteins is still unknown.

W.G. Robertson: To what extent are the observed effects of nephrocalcin and Tamm-Horsfall mucoprotein due to the exceptional conditions of calcium concentration, pH and ionic strength used in these studies?

Author: The conditions of calcium concentration, pH and ionic strength were stressed in order to facilitate the screening of the results (see Deganello, 1991). However, we have compelling evidence that the effects are reproducible even under much less stringent conditions.

W.G. Robertson: In the light of the marked differences in behavior between nephrocalcin and THP, what effect would mixtures of the two proteins have on the crystallization of COD?

Author: We are in the process of carrying out these experiments at present.

J.D. Fairing: It will be helpful to have a drawing representing the designation of wells in the plates.

Author: Please see below; size is 12.6 cm x 8.4 cm.

