The effects of serum and human albumin on calcium hydroxyapatite crystal growth

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The effects of potential serum inhibitors upon the growth of calcium hydroxyapatite (HAP) crystals were studied *in vivo* using a pH-stat system. Whole serum caused a marked decrease in crystal growth in a dose-dependent manner. At a protein concentration of 13 μg/ml, whole serum reduced the initial rate of crystal growth from 84 μmol of KOH/h to 48 μmol of KOH/h. Serum components were separated by ultrafiltration (10000 Da cut-off). The high-molecular-mass fraction containing serum proteins gave an initial rate of crystal growth of 48 μmol of KOH/h compared with 64 μmol of KOH/h given by the low-molecular-mass components. Thus, two-thirds of the inhibitory activity was associated with proteins and other serum macromolecules, whilst the remainder of the activity was associated with the low-molecular-mass components. Albumin-depleted serum showed an initial rate of crystal growth of 59 μmol of KOH/h, whilst albumin purified by affinity chromatography gave an initial rate of crystal growth of 56 μmol of KOH/h at the same protein concentration. Albumin, therefore, not only accounts for half of the protein concentration in serum, but also contributes half of the inhibitory activity of the high-molecular-mass fraction. Heat denaturation of albumin dramatically enhanced the inhibition of HAP seeded growth with the initial rate of crystal growth falling to 27 μmol of KOH/h after treatment compared with 62 μmol of KOH/h before denaturation. Isoelectric focusing indicated that the tertiary and secondary structure, and hence the distribution of surface charge of albumin, are altered by heat denaturation. Gels showed a mixture of species with isoelectric points ranging from 6.0 to 5.0 compared with the native protein value of 4.7. These data suggest that adsorption of serum proteins to the growing HAP crystals is one mechanism of growth inhibition. It is also clear that the most abundant serum protein, albumin, is an important mediator of this process.

INTRODUCTION

The fundamental process of biological mineral deposition is under chemical control, with nucleation and growth of crystals energetically regulated by levels of saturation and local concentrations of co-precipitating ions (Mann, 1988). Nucleation and growth are also controlled by specific interactions of the precipitating mineral phases with organic and/or inorganic molecules, which function as promoters or inhibitors of crystal growth. This leads to the selective deposition of specific structural and morphological mineral phases (Weiner, 1986; Addadi & Weiner, 1985). The specificity of the mineral phase is thus determined by the type of promoter/inhibitor, the concentration of promoter/inhibitor and temporal control of the transport of all of these molecules to the site of mineralization.

A number of macromolecules which inhibit crystal growth have been identified and characterized in human body fluids. For example, salivary statherin (Schlesinger & Hay, 1977; Aoba *et al.*, 1984) and proline-rich peptides secreted by the parotid gland (Oppenheim *et al.*, 1971) function as inhibitors of calcium phosphate formation in saliva. In urine, calcium oxalate monohydrate precipitation is controlled by a 'glycoprotein-crystallization' inhibitor (Nakagawa *et al.*, 1983; Worcester *et al.*, 1987). Calcium carbonate formation in pancreatic secretions is regulated by an acidic phosphoprotein (de Caro *et al.*, 1984). A number of macromolecular complexes which exhibit inhibitory activity *in vitro* have also been isolated from connective tissues (Hunter *et al.*, 1985; Fisher & Termine, 1985; Chen & Boskey, 1986; Boskey, 1989). In comparison to these body fluids, human serum has not been characterized to the same extent. Plasma ultrafiltrate containing low-molecular-mass inorganic ions has been studied in detail and citrate, magnesium and pyrophosphate have been shown to inhibit HAP precipitation (reviewed in Fleisch, 1981; Rufenacht & Fleisch, 1984; Meyer & Fleisch, 1984*a*,*b*; Eidelman *et al.*, 1988). However, despite the identification of a range of biological inhibitors, there is limited knowledge of the precise mechanisms by which the process of crystal growth is inhibited by these macromolecules (Williams & Sallis, 1979, 1982). The present study therefore aimed to identify the macromolecules of serum responsible for inhibition/promotion of HAP crystal growth, using a pH-stat assay of seeded crystal growth.

Preliminary work from this unit has suggested the existence of inhibitors of HAP crystallization in serum and synovial fluid. Sera from patients with osteoarthritis, commonly associated with crystals, were compared with sera from patients with rheumatoid arthritis in the pH-

Abbreviations used: HAP, hydroxyapatite; PBS, phosphate-buffered saline; R_0, initial rate of crystal growth.

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stat crystal growth assay, and both groups showed similar inhibitory potential. The synovial fluid from these arthritic patients also caused inhibition of crystal growth, but in contrast to serum showed a wide range of inhibitory potential (Campion *et al.*, 1988).

Numerous rheumatic diseases involve the deposition of inorganic crystalline phases, including hydroxyapatite, in articular tissues which may lead to chronic joint damage and disability or acute inflammatory responses (Dieppe *et al.*, 1988; Schumacher, 1988). The mechanisms for the formation of these pathological mineral deposits are poorly understood (Smith, 1982). In the long term, an understanding of the molecular mechanisms is vitally important for the development of rational approaches to prevention and treatment of pathological mineral deposition (Dieppe & Calvert, 1983).

Our hypothesis is that normal body fluids contain macromolecular inhibitors of crystal growth which are depleted, ineffective or absent in regions of ectopic mineral deposition. This work was undertaken to give detailed information about the growth of HAP in the presence of normal human serum and to identify the macromolecules that influence crystal growth. These data from normal human sera are an essential prerequisite for testing our hypothesis during comparisons with pathological sera and synovial fluids (Campion *et al.*, 1988).

MATERIALS AND METHODS

Preparation of seed crystal

Synthetic HAP used to seed the growth of crystals in solution was prepared by slowly titrating orthophosphoric acid into a suspension of calcium oxide (20 g, prepared by heating calcium carbonate at 1000 °C for 24 h) to a Ca/P final molar ratio of 1.67 (McDowell *et al.*, 1977). The 1 litre suspension was attached to a reflux condenser, kept under a nitrogen atmosphere, stirred continuously and maintained at 90 ± 1 °C for 48 h. The precipitate was allowed to settle, the liquid phase was removed by centrifugation and the precipitate was washed three times by stirring in 2 litres of distilled water, settling and centrifuging, then similarly washed in 1 mM-orthophosphoric acid and dried at 110 °C for 24 h under nitrogen. This HAP showed sharp i.r. and X-ray diffraction patterns characteristic of well-crystallized HAP without carbonate. The crystal mass was crushed, sieved and those particles between 250 μm and 750 μm collected. The specific surface area of these crystals was measured by the modified 'BET' method (Brunauer *et al.*, 1938) using nitrogen adsorption after degassing at 120 °C and was $30.8 \pm 0.6\ m^2/g$ ($n = 3$; Area-meter, Ströhlein Gmbh & Co.).

Preparation of solutions

All solutions were made with reagent-grade chemicals (BDH Ltd.) using distilled water. Solutions supersaturated with respect to HAP were prepared from stock solutions and mixed to final concentrations of 1.97 mM-$CaCl_2$, 0.96 mM-KH_2PO_4 and 150 mM-KCl, to mimic the concentrations found in serum (Nancollas & Tomazic, 1974; Eidelman *et al.*, 1988). KCl, an inert electrolyte, was included to maintain ionic strength and was essentially constant during the experiment. The saturation as 19.52 with respect of HAP, 2.52 with respect to octacalcium phosphate and 0.77 with respect to dicalcium phosphate dihydrate by using the computer program of Shellis (1988). For serum, the ratios are 14.0–15.0 for HAP and 1.8–2.8 for octacalcium phosphate (Eidelman *et al.*, 1988).

A water-jacketed glass vessel was used for the precipitation reactions. The metastable calcium phosphate solutions, 120 ml, were maintained at 37 °C, continually stirred using a magnetic stirrer and were purged with nitrogen to exclude carbon dioxide. The pH of the reaction solution was measured *in situ* with a combination glass electrode (Metrohm AG) and adjusted to pH 7.40 by addition of 25 mM-KOH using a pH-stat assembly (Metrohm Combititrator, Metrohm AG, Herisau, Switzerland). The solution was maintained at pH 7.40 during crystal growth and the volume of alkali added recorded continuously (Nancollas & Mohan, 1970; Moreno & Varughese, 1981). The control experiments began with the addition of 15 mg of HAP seed crystal suspended in 0.5 ml of HAP-saturated distilled water, had nothing else added and ran for 60 min. The final precipitate was collected by filtration onto 0.22 μm filters (Millipore Ltd.) and dried at 37 °C for 60 min. Samples under investigation were added to the reaction solution in 0.2 ml of phosphate-buffered saline (PBS, Oxoid Ltd.) during the initial pH adjustment after the solution had been adjusted to greater than pH 7.00. The addition of 0.2 ml of PBS alone to the reaction solution showed identical growth kinetics to the control reaction. Measurements were repeated a minimum of three times and a maximum of seven times. The S.D. of the mean and % inhibition of crystal growth were calculated. The latter values varied over the range 1.5–4%.

Blue-Sepharose chromatography

Serum from a healthy individual (2 ml) was applied, in PBS, to a column of Blue-Sepharose (Pharmacia Ltd.; 30 ml, 15 mm diameter, flow rate 30 ml/h) equilibrated in PBS. The absorbance was monitored at 280 nm and fractions collected. The single peak of absorbance representing the unbound proteins was collected. Proteins adsorbed to the column were eluted with PBS containing 1.5 M-KCl (Affinity Chromatography booklet, Pharmacia Ltd.) and dialysed against PBS for a total of 16 h at 4 °C to remove KCl. Both fractions were concentrated by vacuum dialysis (collodion bags, Sartorius GmbH) to a protein concentration of 80 mg/ml, as measured by a dye-binding assay standardized against human serum albumin (BioRad Ltd.). The protein profiles of the two fractions were examined by Coomassie Blue stain of a 12% SDS/polyacrylamide gel (Laemmli, 1970).

Heat-denaturation of albumin

Purified albumin (fraction V, Sigma; 80 mg) was suspended in distilled water (100 ml) and heated at 90 °C in a closed vial for 16 h (Perle-Treves & Addadi, 1988). Aggregated protein was removed by centrifugation (10000 *g* for 15 min), the supernatant collected and the protein content measured by absorbance at 280 nm.

RESULTS

Crystal growth

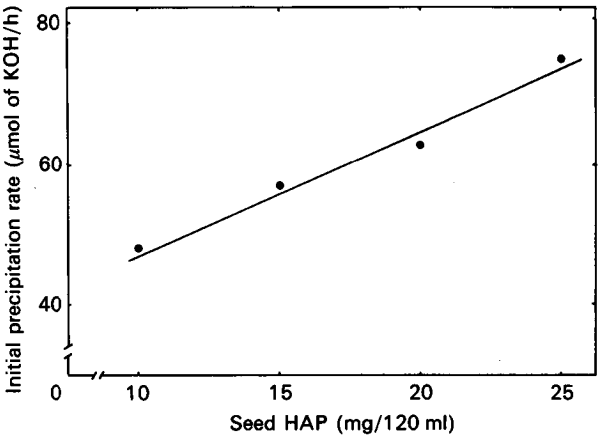

Fig. 1. Initial rates of crystal growth are proportional to the amount of seed HAP used to initiate the precipitation

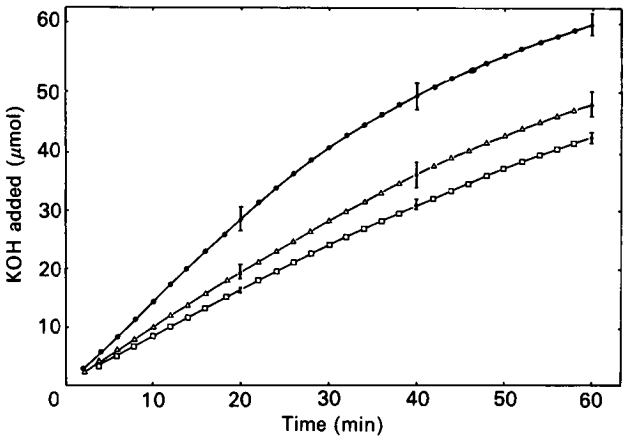

Fig. 2. Comparison of the inhibitory effect of serum and dialysed serum

The effects are shown by the amount of KOH required to maintain a constant pH 7.40 during the precipitation of calcium phosphate at 37 °C in a 120 ml assay. At time zero 15 mg of HAP seed crystals were added to initiate crystal growth. The control experiment did not contain any macromolecules. The protein content of each serum was measured and 16 mg of proteins (~ 0.2 ml) were added to the assay. The values are the means of seven normal sera, three dialysed sera and four controls. The error bars represent S.D. of the mean. Inhibition (%): control (●), 0; serum (□), 43; dialysed serum (△), 32.

and the rate of crystal growth was followed by hydroxyl ion consumption. The quantity of seed HAP crystals added was characterized for each batch of HAP made. Fig. 1 shows that the initial rate of crystal growth (R_0) was linearly related to the surface area of seed HAP available. Since the seed HAP used is constant within any one batch, the weight of seed HAP is proportional to the surface area. Amounts of seed HAP below 10 mg/120 ml solution do not show the same linear relationships. A seed concentration of 15 mg/120 ml solution was chosen for the following experiments and the growth of

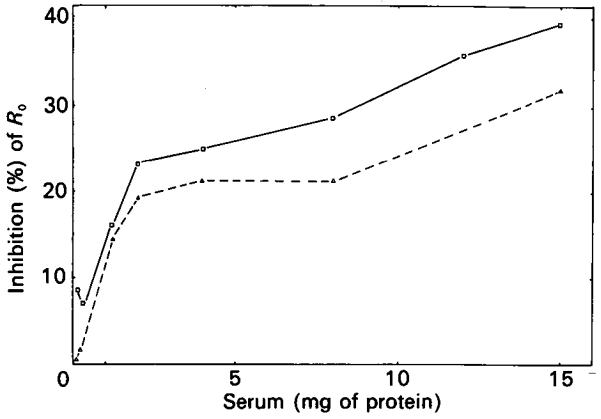

Fig. 3. Dose–responses of serum (□) and dialysed serum (△) on calcium phosphate precipitation

Serial dilutions were made in PBS prior to the addition to the reaction solution and are expressed as the total amount of protein added to the 120 ml assay.

reaction solution (Nancollas & Mohan, 1970; Nancollas & Tomazic, 1974). Initially, the rate of base addition was linear with time and to calculate the initial rate of crystal growth, data points were measured over the first 14 min. Due to the depletion of calcium and phosphate with time the reactions were routinely limited to 60 min. Examination of seed crystals after 60 min gave characteristic patterns for crystalline HAP by i.r. and X-ray diffraction analyses (data not shown). Without the addition of HAP seed crystals no uptake of hydroxyl ions was recorded, indicating that serum alone did not induce nucleation and growth.

Inhibitory effects of serum

The addition of normal serum has an inhibitory effect on HAP crystal growth as shown in Fig. 2. In the control experiment only HAP seed crystal was added and showed an initial growth rate of 84 μmol of KOH/h but after the addition of serum (at a protein concentration at 13 μg/ml), the initial growth rate fell to 48 μmol of KOH/h, a 43 % decrease. Serum was dialysed to remove the low-molecular-mass components and caused the initial rate of crystal growth to fall to 57 μmol of KOH/h, a 32 % decrease from control values.

The inhibition of crystal growth by serum was dose-dependent; as serum was diluted the inhibition of crystal growth decreased (Fig. 3). However, very dilute serum reached a plateau of inhibition during which further dilution does not decrease the inhibition (0.40–0.13 mg of protein/120 ml). This effect could be due to two processes, one operating in a protein-dependent manner until a concentration of 0.4 mg total protein/120 ml was reached and below this value another effect that is not protein-concentration-dependent. To test this hypothesis, the dose–response of dialysed serum was measured and showed inhibition decreasing with decreasing amounts of dialysed serum until it reached the control rate (Fig. 3).

In order to compare directly the low- and high-molecular-mass components of serum, these were separated by ultrafiltration. Both fractions were able to

Table 1. Comparison of the low- and high-molecular-mass components of serum on HAP precipitation

Serum (2 ml) was fractionated by ultrafiltration through a membrane with a 10000 Da cut-off (Amicon Microconcentrator-10). The fractions were made up to equal volumes with PBS (2 ml) and tested for their inhibitory activity by inclusion of 0.2 ml in the seeded crystal growth assay. The initial rates of crystal growth, R_0, were calculated as described in the Results section.

Sample	R_0 (μmol of KOH/h)	Inhibition (%)
Control	79.5	0
Serum	43.5	45
Ultrafiltrate	64.0	19
Retentate	48.0	40

Table 2. Inhibitory effect of purified human serum albumin and albumin-depleted serum

Serum was fractionated on Blue-Sepharose which effectively removes albumin and very few other proteins. The two fractions were assayed at equal protein concentrations of 20 mg/120 ml reaction volume.

Sample	R_0 (μmol of KOH/h)	Inhibition (%)
Control	81.0	0
Flow-through	58.5	28
Eluate	55.5	32

inhibitory as the ultrafiltrate containing the low-molecular-mass components.

Effects of albumin

Since half of the protein in serum is a single species, albumin, its effect upon HAP crystal growth was investigated. Purified albumin (167 μg/ml) added to the pH-stat assay gave an initial rate of crystal growth of 62 μmol of KOH/h. Compared to a control rate of 81 μmol of KOH/h this is an inhibition of 24%. Inhibition of HAP crystal growth by purified human serum albumin also showed a dose–response dependent upon protein concentration (results not shown). However, purified albumin does not wholly represent albumin found *in vivo*, which acts as a transport protein and is complexed to many other serum molecules (Peters, 1975; Kragh-Hansen, 1981). Albumin was therefore removed from serum by dye-ligand chromatography using Blue-Sepharose. Polyacrylamide gel electrophoresis and immunoelectrophoresis showed that albumin was not detectable in the fraction that did not bind to the matrix. The serum fraction eluted from the matrix by high-salt buffer was found to contain albumin and trace amounts of other proteins (e.g. α_2-macroglobulin). The albumin-depleted serum and the albumin-enriched fraction were tested for their ability to inhibit HAP crystal growth, and at equal protein concentrations showed similar inhibitory

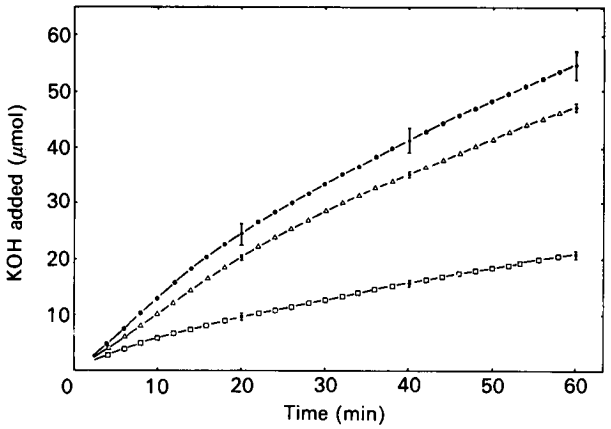

Fig. 4. Comparison of the inhibitory effects of human serum albumin and heat-denatured albumin

Each point represents the mean of triplicate samples and the S.D. values are shown by the vertical bars. Each assay contained 16 mg of protein resuspended in 0.2 ml of PBS. Inhibition (%): control (●), 0; albumin (△), 24; heat-denatured albumin (□), 83.

structure to inhibition of HAP crystal growth by albumin was tested. A solution of purified albumin was denatured by heating and its effect upon HAP crystal growth compared with native albumin in the pH-stat assay (Fig. 4). Denatured albumin reduced the initial rate of crystal growth to 27 μmol of KOH/h, a much bigger decrease than native albumin at 62 μmol of KOH/h. When compared to the control, 81 μmol of KOH/h, these show 83% and 24% inhibition of HAP crystal growth respectively. The native albumin had an isoelectric point (pI) of 4.7 in contrast to the heat-denatured albumin which showed a range of species with pI values ranging from 6.0 to 5.0 (Phast-gel 3-9, Phast System, Pharmacia Ltd.).

DISCUSSION

These data demonstrate that human serum has a significant effect upon HAP crystal growth in the *in vitro* pH-stat assay system. The removal of the low-molecular-mass components, mainly inorganic ions, from serum reduces but does not abolish the inhibitory effect (Fig. 2 and Table 1). The low-molecular-mass inorganic ions may be responsible for the inhibition when serum is very dilute, < 8 μg of protein/ml (Fig. 3). The organic macromolecules of serum play a more important role in controlling the nucleation and growth of HAP than the inhibitory inorganic ions. This was also the conclusion of another study by Meyer & Fleisch (1984*b*) using an assay at constant supersaturation. *In vivo*, organic and inorganic molecules coexist both in serum and in bone mineral and so their effects also depend upon interactions with one another as well as interactions with HAP crystals. During pathological mineralization specific interactions between crystals and biological macromolecules may be significant in controlling the development of crystal deposition diseases (Boskey *et al.*, 1981, 1983; Anderson, 1983; Perle-Treves & Addadi, 1988).

Previous studies using human and rat sera fractionated

1984*a*). However, no further analyses of these protein fractions were undertaken to identify the macromolecules involved. The present study has clearly shown that whole serum can inhibit the seeded growth of HAP (Fig. 2). Affinity-purified human serum albumin has the capacity to inhibit HAP precipitation equal to that of the remaining proteins (see Table 2). In view of these results, the crystal–protein interactions which might mediate the observed inhibition of HAP growth by albumin were investigated.

Terkeltaub *et al.* (1988) have shown that when HAP crystals are incubated in whole human serum a number of proteins are adsorbed and that albumin was a significant component of the adsorbed species. The adsorption of proteins and polypeptides onto HAP has been correlated with the presence of specific functional groups in the organic molecule and specific adsorption sites on the HAP (Williams & Sallis, 1979, 1982; Fujisawa *et al.*, 1986, 1987). From other studies of HAP inhibitors, it is known that, besides functional groups, the structural conformation of the inhibitor molecule is an important determinant for potency (Moreno *et al.*, 1984; Williams & Sallis, 1982; Romberg *et al.*, 1985, 1986; Myers, 1987; Schlesinger *et al.*, 1988). The changes in the surface charge of albumin, demonstrated by changes in the pI, allow increased interactions of the protein with HAP crystals and hence increased inhibition of crystal growth. In a study of serum albumin binding to HAP, Hlady & Füredi-Milhofer (1978) concluded that mutual interaction depended upon surface charge and was increased by a fall in pH, temperature or ionic strength. They also suggested that the conformation of albumin at the protein–crystal interface may not be the same as in solution, with adsorbed albumin unfolding relative to its native state. In the assay, heat-denatured albumin proved to be a three-fold more potent inhibitor of HAP growth than native albumin (Fig. 4), which shows that the structural conformation of this protein is critical in mediating the interactions of the protein with the growing crystals. Although the three-dimensional structure of albumin is not well defined, there is clear evidence that the single polypeptide is ordered into 18 α-helices and that these are aligned into three parallel cylindrical domains (Bloomfield, 1966; Brown, 1977*b*; Kragh-Hansen, 1981). Heat-denaturation induces a partial unfolding of the helical conformation (Perle-Treves & Addadi, 1988). The cylindrical domains have a hydrophobic interior and a polar exterior (Brown, 1977*a*) and this alignment of the polar residues would favour electrostatic interactions between the charged domains on the protein and the exposed surfaces of HAP crystals. By binding to the crystal surface where growth is taking place, the adsorbed molecules would inhibit growth. The question which remains to be answered is whether this inhibitory effect results from interactions between specific molecular motifs in the crystal faces and functional groups within the protein (Moreno *et al.*; 1984; Williams & Sallis, 1982; Perle-Treves & Addadi, 1988) or whether the inhibition arises merely from less specific crystal–protein interactions.

Transmission electron microscope analyses of HAP crystals grown in the presence of human serum albumin indicate that albumin-mediated aggregation may be partly responsible for the observed growth inhibition

crystal faces (B. Heywood & J. Garnett, unpublished work). It is interesting to note that the interaction of human serum albumin with another biologically important mineral, monosodium urate monohydrate, is highly specific. Using immunolocalization, it has been shown that albumin binds selectively to the {110} face of this monoclinic crystal (Perle-Treves & Addadi, 1988).

In the present study we have investigated the possible role of serum components in the control of HAP crystal growth and demonstrated that a key serum component acts as a potent inhibitor of this process. Abnormal tissue mineralization underlies a wide spectrum of pathological disorders and the mineral phases associated with such conditions have been characterized. Associated risk factors which influence their occurrence have been identified, such as increasing age and tissue damage (Dieppe & Calvert, 1983).

A range of normal and apatite-containing tissues have been analysed and a unique set of proteolipids, proteolipid–phospholipid and calcium–phospholipid–phosphate complexes have been identified (Boskey *et al.*, 1982; Wuthier, 1982; Boyan, 1986). These complexes will nucleate HAP from metastable solutions, indicating a potentially important role in biomineralization (Boskey & Posner, 1977). However, it has also been demonstrated that the acidic phospholipid, phosphatidylserine, will inhibit HAP growth when added to metastable calcium phosphate solutions (A. L. Boskey, personal communication); thus the role of phosphorylated membrane components associated with mineralized tissues is unclear and there remains an incomplete understanding of the processes controlling crystal nucleation and growth in pathological mineralization. Knowledge of the molecular mechanisms directing crystal growth and also crystal–protein interactions will lead to a better understanding of the physiological aberrations which induce pathological crystallization and provide a base for the design of therapeutic strategies in the treatment and management of crystal deposition diseases.

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