MINERALIZATION BY INHIBITOR EXCLUSION:
THE CALCIFICATION OF COLLAGEN WITH FETUIN

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One of our goals is to understand the mechanisms that deposit mineral within collagen fibrils, and as a first step we recently determined the size exclusion characteristics of the fibril. This study revealed that apatite crystals up to 12 unit cells in size can access the water within the fibril while molecules larger than a 40 kDa protein are excluded. We proposed a novel mechanism for fibril mineralization based on these observations: that macromolecular inhibitors of apatite growth favor fibril mineralization by selectively inhibiting crystal growth in the solution outside of the fibril.

To test this mechanism, we developed a system in which crystal formation is driven by homogeneous nucleation at high calcium phosphate concentration and the only macromolecule in solution is fetuin, a 48 kDa inhibitor of apatite growth. Our experiments with this system demonstrate that fetuin determines the location of mineral growth: in fetuin’s presence mineral grows exclusively within the fibril while in its absence mineral grows in solution outside the fibril. Additional experiments show that fetuin is also able to localize calcification to the interior of synthetic matrices that have size exclusion characteristics similar to those of collagen, and that it does so by selectively inhibiting mineral growth outside of these matrices.

We term this new calcification mechanism ‘mineralization by inhibitor exclusion’: the selective mineralization of a matrix using a macromolecular inhibitor of mineral growth that is excluded from that matrix. Future studies will be needed to evaluate the possible role of this mechanism in bone mineralization.

The type I collagen fibril plays several critical roles in bone mineralization. The mineral in bone is located primarily within the fibril (1-6), and during mineralization the fibril is formed first and then water within the fibril is replaced with mineral (7,8). The collagen fibril therefore provides the aqueous compartment in which mineral grows. We have recently shown that the physical structure of the collagen fibril plays an important additional role in mineralization: the role of a gatekeeper that allows molecules smaller than a 6 kDa protein to freely access the water within the fibril while preventing molecules larger than a 40 kDa protein from entering the fibril (9).

Molecules too large to enter the collagen fibril can have important effects on
mineralization within the fibril. We have suggested that large inhibitors of apatite growth can paradoxically favor mineralization within the fibril by selectively preventing apatite growth in the solution outside of the fibril (9). We have also proposed that large nucleators of apatite formation may generate small crystals outside the collagen fibril and that some of these crystals can subsequently diffuse into the fibril and grow (9). Because the size exclusion characteristics of the fibril allow rapid penetration of molecules the size of a 6 kDa protein, apatite crystals up to 12 unit cells in size should in principle be able to freely access all of the water within the fibril (9).

We subsequently tested these hypotheses for the role of large molecules in fibril mineralization by determining the impact of removing fetuin on the serum-driven calcification of collagen fibrils (10). Fetuin is the most abundant serum inhibitor of apatite crystal growth (11,12), and with a molecular weight of 48 kDa fetuin is too large to penetrate the collagen fibril (9). Fetuin is also termed fetuin-A (to distinguish it from a recently discovered homologue, fetuin-B (13)) and is sometimes called α2-HS glycoprotein in humans. Our working hypothesis was that fetuin is required for the serum driven calcification of a collagen fibril, and that its role is to favor calcification within the collagen fibril by selectively preventing apatite crystal growth in the solution outside the fibril.

The results of this study demonstrate that removing fetuin from serum eliminates the ability of serum to induce the calcification of a type I collagen matrix, and that adding purified fetuin to fetuin-depleted serum restores this activity (14). This study further shows that a massive mineral precipitate forms during the incubation of fetuin-depleted serum, but not during the incubation of serum containing fetuin (14). These observations are consistent with the hypothesis that a large serum nucleator generates apatite crystals in the solution outside of the collagen fibril, some of which penetrate into the aqueous interior of the fibril (14). Since fetuin can only trap those nuclei that it can access, the crystal nuclei that penetrate the fibril grow far more rapidly than those nuclei trapped by fetuin outside of the fibril, and the collagen fibril therefore selectively calcifies.

The goal of the present experiments was to further understand the role of fetuin in the calcification of type I collagen fibrils. To accomplish this goal, we developed a system in which crystal formation is driven by homogeneous nucleation at high calcium phosphate concentrations, and the only macromolecule in the solution is fetuin. This system allowed us to probe the impact of fetuin and only fetuin on the location and extent of collagen calcification.

Because fetuin is the subject of this study, it is useful to review briefly its occurrence and calcification-inhibitory activity. Fetuin is a 48 kDa glycoprotein that is synthesized in the liver and is found at high concentrations in mammalian serum (15,16) and bone (17-22). The serum fetuin concentration in adult mammals ranges from 0.5 to 1.5 mg/ml, while the serum fetuin concentration in the fetus and neonate is typically far higher (16). Fetuin is also one of the most abundant non-collagenous proteins found in bone (17-22), with a concentration of about 1mg fetuin per g bone in rat (21), bovine (17), and human (19,23) bone. In spite of the abundance of fetuin in bone, however, it has not been possible to demonstrate the synthesis of fetuin in calcified tissues, and it is therefore presently thought that the fetuin found in bone arises from hepatic synthesis via serum (20,22). This view is supported by the observation that fetuin binds strongly to apatite, the mineral phase of bone, and is
selectively concentrated from serum onto apatite (18).

In vitro studies have demonstrated that fetuin is an important inhibitor of apatite growth and precipitation in serum containing increased levels of calcium and phosphate (12), and that targeted deletion of the fetuin gene reduces the ability of serum to arrest apatite formation by over 70% (11). More recent studies have shown that a fetuin-mineral complex is formed in the course of the fetuin-mediated inhibition of apatite growth and precipitation in serum containing increased calcium and phosphate (24, 25). Purified fetuin also potently inhibits the growth of apatite crystals from supersaturated solutions of calcium phosphate (12) (24). In solutions in which a decline in calcium occurs within minutes due to spontaneous formation of apatite crystals, the presence of added fetuin sustains elevated calcium levels for at least 24 hours (24).

EXPERIMENTAL PROCEDURES

Materials. Male albino rats (Sprague-Dawley derived) were purchased from Harlan Labs; Alizarin red S, bovine fetuin, acrylamide, and bisacrylamide were purchased from Sigma; and Sephadex G25 and G75 were obtained from Pharmacia (Piscataway, NJ).

Tibias were dissected from 22-day-old rats and cut to obtain a 1cm section of the tibia midshaft as described (26). Bovine bone sand was prepared from the midshaft region of bovine tibias using procedures that have been described previously [Hale, 1991 #48]; the median diameter of the bone sand was 0.5mm. Rat tibias and bovine bone sand were both demineralized for 72h at room temperature in 0.5M EDTA pH 7.5 using a 300 fold molar excess of EDTA to mineral calcium, washed exhaustively with ultra pure water, dried, and stored at –20ºC until use. Tendons were obtained from the tails of 40-day-old rats as described (26). Four mg samples of dry tendon or demineralized bone were re-hydrated by overnight equilibration in ultra pure water before use. Chondroitin sulfate A (Bovine trachea) was purchased from Calbiochem, dialyzed extensively against 50 mM NH4HCO3 using a 100 kDa MWCO dialysis membrane (Spectra/Por Biotech), and freeze dried. Poly-L-glutamic acid (50 – 100 kDa) was obtained from Sigma. The UCSD Animal Subjects Committee approved all animal experiments.

Biochemical analyses. The procedures used for Alizarin red staining have been described (27). For histological analyses, tibias were fixed in 100% ethanol for at least 1 day at room temperature; San Diego Pathology Inc. (San Diego, CA) sectioned and von Kossa stained the tibias. For quantitative assessment of the extent of calcification, Alizarin red stained matrices and precipitates formed outside the matrix were extracted for 24h at room temperature with 1 ml of 0.15 M HCl, as described (28). Calcium levels in calcification solutions and in the acid extracts of tissues and precipitates were determined colorimetrically using cresolphthalein complexone (JAS Diagnostics, Miami FL) and phosphate levels were determined colorimetrically as described (29).

In order to compare the ability of fetuin to penetrate synthetic matrices, each matrix was equilibrated overnight with a 5 mg/ml solution of fetuin and then stained for protein with Coomassie Brilliant Blue. Sephadex G75 beads and 4% acrylamide gels stained intensely blue, showing that fetuin penetrated both matrices. In contrast, Sephadex G25 beads and 40% acrylamide gels did not stain.

Calcification Procedures. The typical solution used for investigating matrix calcification was prepared at room temperature using a procedure designed to
achieve the near instantaneous mixing of calcium and phosphate and to thereby ensure that subsequent mineral formation occurred by homogenous nucleation in the resulting unstable solution (24). One ml of 0.2M HEPES pH 7.4 containing 10mM CaCl₂ was placed into one 10 X 75mm test tube, and a second 1ml of 0.2M HEPES pH 7.4 containing 10mM sodium phosphate (also pH 7.4) was placed into a second tube. A disposable pipette was then used to withdraw the phosphate solution and to then expel this solution with force into the calcium solution. All HEPES buffer solutions contained 0.02% sodium azide to prevent bacterial growth; the HEPES buffer for all fetuin-containing calcification solutions also contained 5mg bovine fetuin per ml buffer. Unless otherwise stated, the matrices tested using this procedure were added immediately after mixing to achieve the final 5mM calcium and phosphate conditions, and included: a 1cm segment of hydrated, demineralized tibia midshaft from a weanling rat (dry weight about 4 mg); hydrated, demineralized bovine bone sand (4mg dry weight); hydrated rat tail tendons (4mg dry weight); hydrated Sephadex G25 or G75 (4mg dry weight); and single 1x5x5mm segments of 4 or 40% polyacrylamide slab gels (40% is 39.33g acrylamide and 0.67g bisacrylamide per 100ml). To monitor the decrease in calcium due to the formation of mineral, aliquots of the calcification solution were removed at the desired times and centrifuged for 10 seconds to sediment mineral; the supernatant was then diluted 1:4 with 0.2M HEPES pH 7.4 and analyzed for calcium.

To determine the capacity of bone for mineral, 4 mg of demineralized bovine bone sand (dry weight) was added to a 50ml volume of fetuin calcification solution (5mM calcium and phosphate, 0.2M HEPES pH 7.4, 45mM NaHCO₃, 5 mg/ml fetuin, and 0.02% azide) and mixed end over end at room temperature for 2 days. For subsequent re-calcification cycles, the spent solution was replaced with fresh calcification solution and the bone sand was mixed for another 2 days. To determine the importance of demineralization to the capacity of bone for mineral, this experiment was repeated using 18 mg of non-demineralized bone sand, an amount that yields 4 mg of demineralized bone matrix.

For preparation of re-calcified bone matrix for spectroscopic analysis, 4 mg of demineralized bovine bone sand (dry weight) was again added to each of three 50ml volumes of fetuin calcification solution and mixed end over end at room temperature for 2 days. The re-calcified bone sand was dried and ground in an agate mortar; an equivalent amount of non-demineralized bovine boine sand served as a control. The resulting powders were first analyzed using a Scintag SDF 2000 X-ray diffractometer, and a portion of this powder was then analyzed at 4 cm⁻¹ resolution for 256 scans using a Nicolet Magna IR 550 FTIR Spectrometer.

To prepare calcified tendon collagen for scanning electron microscopy, 4 mg of rat tail tendon (dry weight) was added to a 50ml volume of fetuin calcification solution and mixed end over end at room temperature for 2 days. Samples of calcified and non-calcified tendon collagen were washed with 0.05% KOH, dehydrated in ethanol, and dried. The samples were then sputter coated with an ultra thin layer of gold/palladium and examined at 20 kV with an FEI Quanta 600 scanning electron microscope with an Oxford energy dispersive X-ray spectrometer (EDX).

RESULTS

Bone can be re-calcified by using fetuin to selectively inhibit mineral growth outside the collagen fibril. We first
determined whether fetuin is able to selectively favor the re-calcification of the type I collagen fibrils in demineralized bone when crystal nuclei are generated by homogeneous nucleation at high calcium phosphate ion product. The high ion product solution was generated by rapidly mixing equal 1 ml volumes of 10 mM phosphate and 10mM calcium in order to obtain a homogenous solution containing 5mM of each ionic component in a pH 7.4 buffer. Previous studies have shown that a calcium phosphate mineral forms throughout this solution within minutes of mixing, while if fetuin is added prior to mixing there is no visible evidence of mineral formation (12,24). A 1 cm segment of demineralized rat tibia midshaft was added immediately after mixing. In this 2 ml volume, there is only sufficient calcium and phosphate to restore approximately 5% of the mineral that was present in the tibia prior to demineralization.

The rate of mineral formation was monitored by the decline in calcium remaining in solution. As seen in Figure 1, there was no decrease in calcium in solutions containing fetuin but no tibia. This result is consistent with earlier studies (26) and illustrates the ability of fetuin to potently inhibit mineral growth and precipitation. As also seen in Figure 1, if both fetuin and a demineralized tibia are present there is a decrease in solution calcium that begins about 5 hours after addition of the tibia, and solution calcium is reduced by about 4 fold at 8 hours. Chemical analysis showed that the amount of calcium and phosphate incorporated into the tibia at 24h accounted for the decrease in solution calcium and phosphate, and there was no evidence for a calcium phosphate precipitate in the solution outside of the tibia (Figure 2). The re-calcified tibias stained uniformly for calcification with Alizarin red, and von Kossa staining of tibia sections showed that calcification foci are found throughout the bone matrix (not shown).

These experiments were repeated using solutions of the same composition but lacking fetuin in order to confirm the role of fetuin in the re-calcification of demineralized tibias. In agreement with earlier studies (24), in the absence of fetuin a finely dispersed mineral precipitate formed within minutes of mixing to create 5mM calcium and phosphate, and solution calcium levels fell 5 fold within 2 hours of mixing (Figure 1). The presence of a demineralized tibia had no significant impact on the rate of calcium loss from solution in this experiment (Figure 1). After 24 hours incubation in the solution lacking fetuin, chemical analysis showed that most of the mineral present was in a precipitate in the solution outside of the tibia, not within the tibia (Figure 2), and the tibia did not stain with Alizarin red or von Kossa (not shown).

These observations clearly show that the presence of fetuin in an unstable, supersaturated solution containing 5 mM calcium and phosphate determines the location of the calcium phosphate mineral growth: in the absence of fetuin, mineral growth occurs primarily in the solution outside bone collagen while in the presence of fetuin, mineral growth occurs almost exclusively within bone collagen.

**Determination of the amount of mineral that can be deposited in bone collagen by using fetuin to selectively inhibit mineral growth outside the collagen fibril.** We next investigated the capacity of bone collagen to take up mineral using the fetuin re-calcification procedure. Ground bone was used for this test rather than a tibia in order to increase the ratio of matrix surface to volume and thereby enhance the diffusion of calcium, phosphate, or small crystals into collagen. The volume of the fetuin-containing re-calcification solution was increased to 50 ml so that the
calcium in the re-calcification solution (250 µmol) would exceed the calcium originally found in the bone matrix (114 µmol). Finally, some of the samples were subjected to as many as three consecutive re-calcification cycles, each in fresh 50 ml volumes of re-calcification solution.

The first experiment examined the capacity of demineralized bone to take up mineral during three successive re-calcification cycles. As can be seen in Figure 3, the greatest increase in mineral occurred in the first re-calcification cycle, and declined markedly by the third. At this point, the amount of calcium and phosphate introduced into demineralized bone was about 70% of that found in the adult bovine bone prior to demineralization.

The second experiment showed that a single re-calcification cycle does not significantly increase the mineral content of non-demineralized bone (Figure 3). This observation shows that the incorporation of mineral into bone using this procedure requires prior demineralization.

Evidence that the mineral in re-calcified bone collagen is similar to bone mineral. We used several methods to assess the nature of the calcium phosphate mineral incorporated into demineralized bone by this procedure. The results of these measurements revealed that the mineral in re-calcified bone is similar to the mineral found in bone prior to demineralization: 1. The molar calcium to phosphate ratios calculated from the data in Figure 3 range from 1.68 ± 0.03 for the first re-calcification cycle to 1.66 ± 0.03 for the second and third cycles. These ratios are not significantly different from the ratios calculated from the Figure 3 data for non-demineralized bone, 1.66 ± 0.02 and 1.64 ± 0.03. 2. The powder X-ray diffraction (XRD) spectrum obtained for demineralized bone after one re-calcification cycle is comparable to the spectrum obtained for bone prior to demineralization (Figure 4) and the diffraction peaks seen in both spectra are in the positions expected for synthetic hydroxyapatite crystals (30). 3. The fourier transform infrared (FTIR) absorbance spectra obtained for demineralized bone after one re-calcification cycle is comparable to the spectrum obtained for bone prior to demineralization (Figure 4).

Further characterization of the role of fetuin in collagen calcification. In the above experiments we have consistently used a 5 mg/ml fetuin concentration to inhibit mineral growth in the solution outside the collagen fibril. This fetuin concentration is lower than that found in fetal bovine serum (20 mg/ml)(16) and substantially higher than the mean serum fetuin level found in adult human serum (about 0.9 mg/ml)(31). Additional experiments were therefore carried out to determine the dependence of collagen calcification on fetuin concentration in this model system.

Figure 5 shows that fetuin concentrations of 1 to 10 mg/ml are able to selectively calcify collagen in a solution that initially contains 5 mM calcium and phosphate, with no evidence for mineral deposition in the solution outside the collagen fibril. The location of mineral deposition shifts from the collagen fibril to the solution outside the fibril as fetuin concentrations are reduced below 1 mg/ml, with the cross over between 0.25 and 0.1 mg/ml fetuin.
Since the dose of fetuin needed to selectively calcify collagen may depend on the rate of crystal formation, we carried out an additional experiment to determine the dose of fetuin required to calcify collagen when the concentrations of calcium and phosphate are reduced to 4 mM. As can be seen in Supplemental Figure A, reducing the concentration of calcium and phosphate from 5 mM to 4 mM decreased the minimum amount of fetuin needed to achieve the selective calcification of collagen from 1 mg/ml to 0.1 mg/ml.

In all of the above experiments we have added the collagen matrix immediately after mixing to create the solution containing 5 mM calcium and phosphate. The prompt addition of collagen after mixing may not be necessary, since the data in Figure 1 show that fetuin maintains a high concentration of calcium for at least 24 hours. To test this possibility, we examined the impact of delaying collagen addition on its calcification. As shown in Figure 6, collagen is still efficiently calcified even when it is added 10 hours after mixing to create the 5 mM calcium and phosphate. There is a significant reduction in calcium and phosphate incorporation when the collagen is added 24 hours after mixing (p < 0.01; Figure 6), and the total amount of mineral incorporated is reduced about 25%.

An experiment was carried out in order to determine whether other inhibitors of calcium phosphate mineral formation that are too large to penetrate the collagen fibril have a similar ability to selectively calcify collagen. As seen in Supplemental Figure B, chondroitin sulfate (MW > 100 kDa) is unable to drive the selective calcification of collagen, while poly-L-glutamic acid (MW > 50 kDa) achieved about 25% of the calcification seen with the same concentration of fetuin. There was a mineral precipitate in the solution outside the collagen fibril with both chondroitin sulfate and poly-L-glutamate but not with fetuin (not shown), which indicates that failure of these inhibitors to selectively calcify collagen may be due to a reduced ability to retard mineral growth in the solution outside the collagen fibril.

We have previously hypothesized that calcification inhibitors that are small enough to penetrate the collagen fibril will prevent mineral growth inside the fibril, not selectively calcify the fibril (9). We tested this hypothesis using bone Gla protein (BGP; osteocalcin), a 6 kDa inhibitor of apatite growth (32) that is able to rapidly penetrate all of the water within the collagen fibril (9). The results of this experiment show that BGP prevents mineral formation inside the collagen fibril (Supplemental Figure B) and in the solution outside the fibril (not shown). The calcification of collagen in solutions containing fetuin is also prevented by BGP (not shown).

Tendon collagen can be calcified by using fetuin to selectively inhibit mineral growth outside the collagen fibril. We next determined whether fetuin is also able to selectively favor calcification of the type I collagen fibrils of rat tail tendon, a tissue that does not normally calcify in vivo. Segments of tendon were added to calcification solutions identical to those used for the re-calcification of demineralized tibias, and tendon calcification was evaluated using the same procedures. There was again a decrease in solution calcium that began 5 hours after addition of the tendons, and solution calcium was reduced 4-fold by 8 hours (not shown). After 24 hours, chemical analysis showed that the amount of calcium and phosphate found within the tendons accounted for the decrease in solution calcium and phosphate, with no evidence for the precipitation of a calcium phosphate mineral in the solution outside the tendons (Figure 7). The calcified tendons stained uniformly for calcification...
with Alizarin red, and von Kossa staining of tendon sections showed that the calcification consisted of numerous calcification foci scattered within the collagen matrix (Supplemental Figure C). These experiments were repeated using solutions of the same composition but lacking fetuin in order to confirm the essential role of fetuin in the calcification of tendon collagen. After 24 hours incubation, chemical analysis showed that all mineral was in a precipitate outside of the tendon collagen, not within the collagen (Figure 7), and the tendons did not stain with Alizarin red or von Kossa (Supplemental Figure C).

Evidence that the mineral in calcified tendon is located within the collagen fibers.

We used scanning electron microscopy to determine whether the mineral in tendon collagen that has been calcified by these procedures is indeed within collagen fibers. As seen in Figure 8, the incorporation of mineral into tendon did not change the size of the collagen fibers, and there is no evidence for the precipitation of mineral on the fiber surfaces. Elemental analysis of calcified tendon (bottom panels of Figure 8) demonstrated that calcium and phosphate co-localize with the collagen fibers. Electron Dispersive X-Ray (EDX) spectra confirm that calcified tendon collagen contains calcium and phosphate (Supplemental Figure D).

Synthetic matrices that have size exclusion characteristics similar to type 1 collagen can be calcified by using fetuin to selectively inhibit mineral growth outside the matrix. If the role of the type 1 collagen fibril in this calcification mechanism is merely to provide an aqueous compartment that excludes fetuin but not calcium and phosphate, then synthetic matrices that define an aqueous compartment with similar size exclusion characteristics should also calcify in solutions containing fetuin and 5mM calcium and phosphate. Sephadex G25 was chosen for the first test, since the spherical beads of this gel filtration media contain an aqueous volume that excludes fetuin but not calcium and phosphate.

Sephadex G25 was added to calcification solutions identical to those used for the calcification of collagen matrices, and the calcification of Sephadex G25 was evaluated using the same procedures. The results of this experiment show that Sephadex G25 calcifies if fetuin is present: 1. There was a decrease in solution calcium that began 5 hours after addition of Sephadex G25, and solution calcium was reduced 5-fold by 8 hours (Figure 9). 2. Chemical analysis showed that the amount of calcium and phosphate found within Sephadex G25 at 24 hours accounted for the decrease in solution calcium and phosphate, with no evidence for the precipitation of a calcium phosphate mineral in the solution outside the Sephadex G25 beads (Figure 10). 3. Alizarin red staining showed that each bead had numerous mineral foci scattered uniformly throughout the interior of the gel particle (not shown). The results of this experiment also show that fetuin is required for Sephadex G25 calcification: In the absence of fetuin all mineral was in the solution outside of Sephadex G25, not within (Figure 10), and the Sephadex G25 did not stain with Alizarin red (not shown).

We carried out an additional experiment to directly test the hypothesis that fetuin must be excluded from the interior aqueous compartment of a matrix for the matrix to be calcified by these procedures. Sephadex G75 was used for this test, because the well-defined size exclusion characteristics of this matrix predict that fetuin should be able to freely penetrate the interior of the gel bead (a result confirmed here, see Experimental Procedures). The results of this experiment show that
Sephadex G75 fails to calcify in the presence of fetuin: 1. There was no decrease in solution calcium over the 24-hour period of observation (Figure 9). 2. Chemical analysis showed that there was no detectable mineral calcium and phosphate either within Sephadex G75 or in the solution outside of Sephadex (Figure 10). 3. Alizarin red staining showed that none of the Sephadex G75 beads were calcified (not shown).

Essentially identical results were obtained when the above Sephadex experiments were repeated using polyacrylamide gels with different acrylamide concentrations (data not shown). Gels that excluded fetuin (such as 40% acrylamide gels) calcified in the pH 7.4 buffer containing 5 mM calcium and phosphate and 5 mg/ml fetuin, while gels that could not exclude fetuin (such as 4% acrylamide gels) were not calcified. If fetuin was omitted, the same amount of mineral again formed in solution and the gels were not calcified.

DISCUSSION

Our goal in the present experiments was to understand the role of fetuin in the calcification of type 1 collagen fibrils. To accomplish this goal, we developed a system in which crystal formation is driven by homogeneous nucleation at a high calcium phosphate ion product, and the only macromolecule in the solution is fetuin. This system allowed us to probe the impact of fetuin and only fetuin on the location and extent of collagen calcification. The results of these tests demonstrate that fetuin is all that is needed to determine the location of mineral growth: in the presence of fetuin mineral grows within the collagen fibril while in its absence mineral grows in the solution outside of collagen. The resulting calcification reaction is stunningly rapid and extensive: after incubation for just 8 hours the concentration of calcium in the tibia is over 2000-fold higher than the concentration of calcium remaining in solution.

Considering the chemical simplicity of this calcification mechanism, it is extraordinary that the initial, rapid phase of collagen calcification with fetuin achieves a total mineral content approximately 70% of that found in the original bone prior to demineralization after a total calcification interval of just 6 days at room temperature. This is comparable to the amount of mineral introduced into collagen during the primary phase of bone mineralization (33,34). It is also extraordinary that the mineral formed within the collagen has a comparable molar calcium to phosphate ratio, FTIR spectrum, and powder XRD spectrum as bone mineral. The same observations have been made using the chemically identical type 1 collagen fibrils of tendon: There is nothing about demineralized bone collagen that makes this matrix more ‘calcifiable’ than tendon collagen.

We also examined the role of the type 1 collagen fibril. We reasoned that, if the role of the type 1 collagen fibril in this calcification mechanism is merely to provide an aqueous compartment that excludes fetuin but not calcium and phosphate, than a synthetic matrix that contains an aqueous compartment with similar size exclusion characteristics should also be calcified in solutions containing fetuin and 5 mM calcium and phosphate. The results of these tests show that synthetic matrices that exclude fetuin but not calcium and phosphate (e.g., Sephadex G25 beads) do calcify in solutions containing fetuin and 5 mM calcium and phosphate, while synthetic matrices that cannot exclude fetuin (e.g., Sephadex G75) do not calcify. These observations indicate that the role of the collagen fibril in this calcification is indeed to provide an aqueous compartment that excludes fetuin but not calcium and phosphate. Fetuin is able to direct
calcification to the interior of any matrix with size exclusion characteristics similar to collagen by selectively inhibiting mineral growth outside of that matrix.

We have previously suggested that calcification inhibitors that are small enough to penetrate the collagen fibril will prevent mineral growth inside the fibril, not selectively calcify the fibril (9). We have tested this hypothesis using BGP, a 6 kDa inhibitor of apatite growth (32) that is able to rapidly penetrate all of the water within the collagen fibril (9). The results of these experiments show that BGP prevents mineral formation inside the collagen fibril, and does not selectively calcify the fibril. We have also tested this hypothesis using matrix Gla protein (MGP), a potent mineralization inhibitor that is also small enough to penetrate the fibril (9). This test shows that just 20 µg MGP/ml is sufficient to prevent the fetuin-dependent calcification of collagen (Villa and Price, personal observations). These in vitro experiments may explain why the over expression of MGP in bone inhibits collagen calcification in vivo (35), and does not promote it.

The synthesis of new mineralized collagenous materials by using fetuin to selectively inhibit mineral growth outside collagen. The ability to replace the mineral phase of bone using only fetuin, calcium, and phosphate could have several applications in the bone and dental implant field. The mineral in bone could be replaced with a less soluble mineral phase, such as fluorapatite, in order to prolong implant life. Alternatively, agents that promote bone growth, such as strontium, could be incorporated into bone during re-calcification in order to stimulate local bone formation.

The ability to calcify purified type 1 collagen could also have uses. Metallic, plastic, and other non-collagenous devices could be coated with collagen, and the collagen coating could then be calcified by these procedures. This could enhance bonding of the device to bone and thereby increase the lifetime of the implant.

**Mineralization by inhibitor exclusion: a novel method for the creation of new crystalline materials.** It is possible that the principles of matrix mineralization described here are general, and that it may prove feasible to place crystals other than apatite into matrices other than collagen using crystal growth inhibitors other than fetuin. Our experiments indicate that only requirements are a macromolecular crystal growth inhibitor in a solution that would, in the absence of the inhibitor, spontaneously form the crystalline phase, and a matrix that excludes the inhibitor but allows the constituents of the crystal to enter the matrix. The liquid need not be water, the temperature need not be ambient, and the pressure need not be 1 atmosphere. Crystal formation can be directed into spaces defined at the nanometer scale, as shown by the efficient calcification of the 40 nm diameter fibrils of bone collagen, and in spaces pre-determined by the location of the matrix ‘mold’ into which the crystals are deposited. We suggest that this novel procedure for the formation of new crystal-matrix composites be termed ‘mineralization by inhibitor exclusion.’

Although derived from the study of biological systems, it is possible that the principles of mineralization by inhibitor exclusion discovered here may form the basis for the fabrication of useful materials that have no direct relationship to biology.

**Summary and perspective:** In the present study, we have used a solution in which mineral forms rapidly due to the high concentration of calcium and phosphate in order to test the hypothesis that fetuin, a macromolecular inhibitor of apatite growth, favors mineralization of the collagen fibril by selectively inhibiting crystal growth in...
the solution outside of the fibril. In this simplified model system, we demonstrate that fetuin is both necessary and sufficient for calcification of the type 1 collagen fibril.

We term this new calcification mechanism ‘mineralization by inhibitor exclusion’: the selective calcification of the type 1 collagen fibril using a macromolecular inhibitor of mineral growth that is excluded from the fibril. This is the first molecular mechanism of collagen calcification to be demonstrated in vitro and future studies will be needed in order to understand the possible relevance of this mechanism to normal bone mineralization. These include: studies to determine whether the first crystals are deposited in the hole region of the collagen fibril, as is the case in normal collagen calcification (36); investigations to compare the mechanical strength of bone that has been re-calciﬁed by these procedures to that of normal bone; and experiments to determine whether the mineral initially deposited within the collagen fibril by the present mechanism eventually grows into the region between fibrils, resulting in the interfibrillar mineral that has been observed in normal collagen calcification (37,38).

Fetuin is a serum protein that is made by liver, not bone (20,22). If fetuin indeed promotes bone mineralization by the ‘mineralization by inhibitor exclusion’ mechanism, it seems likely that the activity of fetuin in bone mineralization is proportional to its serum concentration. It is therefore of interest to note the two observations that support a link between elevated serum fetuin and increased bone mineralization:

1. Serum fetuin levels are typically higher in early fetal life than in the adult; for example, fetuin levels are about 20 mg/ml in fetal calves (gestational age 90d), 10 mg/ml at birth (gestational age 280d), and 1 mg/ml in adult cows (10,16). These developmental differences in serum fetuin may reflect the need to support a higher rate of bone mineralization in the fetus, since our present study shows that acceleration of mineral formation in vitro increases the amount of fetuin needed to support collagen calcification (Figure 5 and Supplemental Figure A).

2. We have recently shown that higher serum fetuin levels are significantly associated with higher total hip, lumbar spine, and whole body bone mineral density (BMD) among well-functioning community dwelling older women (31). For example, each standard deviation (0.38 mg/ml) higher level of fetuin above the 0.93 mg/ml mean is associated with 0.016 g/cm² higher total hip areal BMD. These observations are consistent with our in vitro evidence that higher fetuin levels drive increased collagen calcification regardless of whether apatite crystals are generated by the serum nucleator (10) or by homogeneous nucleation at high calcium and phosphate (Figure 5).

It is important to emphasize in closing that the calcification of collagen that occurs during normal bone formation is a far more complex process than the simple model system described here, and that there is as yet no direct, in vivo evidence that large inhibitors of apatite crystal growth such as fetuin actually play a role in collagen calcification by selectively inhibiting crystal growth in the solution outside of the fibril. The major value of model systems such as the one described here is not to prove how collagen calcifies in bone, but to identify the possible mechanisms of collagen fibril calcification and so stimulate experiments that test these mechanisms in mineralizing bone.
REFERENCES

FIGURE LEGENDS

Figure 1. The re-calcification of bone by using fetuin to selectively inhibit mineral growth outside the collagen fibril: time course of supernatant calcium. The test matrix was a 1 cm segment cut from the midshaft region of a rat tibia and demineralized in EDTA for 72 hours (26). The solutions for the calcification test were prepared as described (24) and contained 2 ml HEPES pH 7.4 with 5mM calcium and phosphate and either 5 mg/ml fetuin or no fetuin. A single demineralized tibia was added immediately after mixing to create the 5 mM conditions and the solutions were mixed end over end at room temperature; there were three tubes per experimental group. Aliquots of each solution were removed at the indicated times and analyzed for calcium; each time point is the average calcium level in the 3 replicate solutions.

Figure 2. The re-calcification of bone by using fetuin to selectively inhibit mineral growth outside the collagen fibril: analysis for mineral calcium and phosphate. The experiment described in the legend to Figure 1 was terminated at 24h, and the mineral that precipitated outside of the tibia was separated from the tibia. The mineral precipitate and tibia were then both analyzed for calcium and phosphate. The results show the mean and standard deviation of the measurements made on the 3 replicate bone samples at each condition.

Figure 3. Evidence that the capacity of bone collagen for mineral is limited. Either 4 mg of demineralized bone sand or an amount of non-demineralized bone sand with the same collagen content (18 mg) was added to a 50ml volume of 0.2M HEPES pH 7.4 containing 5 mg/ml fetuin, 5mM calcium, and 5mM phosphate, and the solution was mixed end over end at room temperature for 2 days. For subsequent re-calcification cycles, the spent solution was replaced with fresh calcification solution and the bone sand was mixed for another 2 days. The bone sand was then analyzed for calcium and phosphate. The results show the mean and standard deviation of the measurements made on the 3 replicate bone samples at each condition.

Figure 4. The Fourier Transform Infrared (FTIR) and powder X-ray diffraction (XRD) spectra of bone that has been re-calcified by using fetuin to selectively inhibit mineral growth outside the collagen fibril. Demineralized bovine bone sand was re-calcified
with fetuin as described in the Figure 3 legend, and samples of the recalcified bone and of non-
demineralized bone were each ground to a fine powder. The graph shows the FT-IR spectrum of
each sample, and the inset shows the powder X-ray diffraction spectrum. (see Experimental
Procedures for details).

**Figure 5.** The dependence of bone collagen calcification on fetuin concentration when
homogeneous crystal formation is driven by 5 mM calcium and phosphate. Four mg of
demineralized bone sand was added to a 2 ml volume of 0.2M HEPES pH 7.4 containing 5 mM
calcium, 5 mM phosphate, and the indicated concentration of fetuin. The solution was mixed
end over end at room temperature for 2 days, and the bone sand was then analyzed for calcium
and phosphate. (see Experimental Procedures for details).

**Figure 6.** Evidence that fetuin sustains conditions that calcify bone collagen. Two ml
volumes of 0.2M HEPES pH 7.4 were prepared that contained 5 mM calcium, 5 mM phosphate,
and 5 mg/ml fetuin. Four mg of demineralized bone sand was added at the indicated times after
mixing calcium and phosphate. The solution was then mixed end over end at room temperature
for 2 days, and the bone sand was analyzed for calcium and phosphate. The results show the
mean and standard deviation of the measurements made on the 3 replicate bone samples at each
condition. (see Experimental Procedures for details).

**Figure 7.** The calcification of tendon collagen by using fetuin to selectively inhibit mineral
growth outside the collagen fibril: analysis for mineral calcium and phosphate. The
solutions for the calcification test were prepared as described (24) and contained 2 ml HEPES
pH 7.4 with 5mM calcium and phosphate and either 5 mg/ml fetuin or no fetuin. Hydrated rat
tail tendon (4 mg dry weight) was added immediately after mixing to create the 5 mM conditions
and the solutions were mixed end over end for 24h at room temperature; there were three tubes
per experimental group. Mineral that precipitated in the solution outside of the tendon was
separated from the tendon, and the mineral precipitate and tendon were both analyzed for
calcium and phosphate. The results show the mean and standard deviation of the measurements
made on the 3 tendon samples at each condition.

**Figure 8.** Scanning electron microscopy shows that mineral is located within the collagen
fibers of tendon that has been calcified using fetuin. The procedure described in the Figure 3
legend was used to calcify 4 mg of rat tail tendon (dry weight). The calcified collagen was
washed with 0.05% KOH, dehydrated in ethanol, and dried. Samples were then sputter coated
with an ultra thin layer of gold/palladium and examined with a scanning electron microscope at
20 kV. The bottom two panels show the results of the elemental analysis performed on the
60,000 X field immediately above: carbon is green; calcium is blue; phosphorus is red; and areas
containing calcium and phosphorus are purple. (The EDX spectra of these 60,000 X fields are
shown in Figure C.) Bars are 20 µm for the top image, and 1 µm for the bottom 4. (See
Experimental Procedures).

**Figure 9.** The calcification of Sephadex G25 by using fetuin to selectively inhibit mineral
growth outside the gel beads: time course of supernatant calcium. The solutions prepared for
the calcification test contained 2 ml HEPES pH 7.4 with 5mM calcium and phosphate and: fetuin
only; Sephadex G25 only; fetuin plus Sephadex G25; and fetuin plus Sephadex G75. Each
solution was placed into a 10x75mm tube and mixed end over end at room temperature; there
were three tubes per experimental group. Aliquots of each solution were removed at the
indicated times and analyzed for calcium; each time point is the average calcium level in the 3
replicate solutions.
Figure 10. The calcification of Sephadex G25 by using fetuin to selectively inhibit mineral growth outside the gel beads: analysis for mineral calcium and phosphate. The experiment described in the Figure 7 legend was terminated at 24h, the mineral that precipitated outside of the Sephadex was separated from the Sephadex using a 20 micron sieve, and the mineral precipitate and Sephadex were both analyzed for calcium and phosphate. The results show the mean and standard deviation of the measurements made on the 3 replicate Sephadex samples tested at each condition.

SUPPLEMENTAL FIGURES

Figure A. The dependence of collagen calcification on fetuin concentration when homogeneous crystal formation is driven by 4 mM calcium and phosphate. Four mg of demineralized bone sand was added to a 2 ml volume of 0.2M HEPES pH 7.4 containing 4 mM calcium, 4 mM phosphate, and the indicated concentration of fetuin. The solution was mixed end over end at room temperature for 3 days, and the bone sand was then analyzed for calcium and phosphate. (see Experimental Procedures for details).

Figure B. Comparison of the ability of high molecular weight inhibitors of mineral formation to re-calcify bone by selectively inhibiting mineral growth outside the collagen fibril. Four mg of demineralized bone sand was added to a 2 ml volume of 0.2M HEPES pH 7.4 containing 5 mM calcium, 5 mM phosphate, and a 1 mg/ml concentration of fetuin, chondroitin sulfate (MW < 100 kDa), poly-L-glutamic acid (MW < 50 kDa), or bone Gla protein (BGP; MW ~ 6 kDa). The solution was mixed end over end at room temperature for 2 days, and the bone sand was then analyzed for calcium and phosphate. (see Experimental Procedures for details).

Figure C. The calcification of tendon collagen by using fetuin to selectively inhibit mineral growth outside the collagen fibril: Alizarin red and von Kossa staining. Rat tail tendons were calcified as described in the Figure 7 legend. In brief, the calcification solutions contained 2 ml HEPES pH 7.4 with 5mM calcium and phosphate and either 5 mg/ml fetuin or no fetuin. Hydrated rat tail tendon (4 mg dry weight) was added immediately after mixing to create the 5 mM conditions and the solutions were mixed end over end for 24h at room temperature. Tendons were then either stained with Alizarin red or cut in 5 micron sections and stained by von Kossa (stains mineral dark brown). Note that the amount of calcium and phosphate in the 2 ml volume of calcifying solution used in this experiment is only sufficient to introduce a limited amount of mineral into tendon (about 4% of the amount introduced into tendon for the scanning electron microscope analysis shown in Figure 8).

Figure D. Electron Dispersive X-Ray (EDX) spectra demonstrate that calcium and phosphate are in the collagen fibers of tendon that has been calcified using fetuin. These EDX spectra were determined on the same fields shown in the bottom two panels of Figure 8. The peak heights were normalized to Palladium.
Figure 1

Supernatant Calcium, mM

Time after mixing to achieve 5 mM calcium and phosphate, hours

- Fetuin Only
- No Tibia or Fetuin
- Demineralized Tibia plus Fetuin
- Demineralized Tibia Only
Figure 2

Demineralized Bone + Fetuin

Demineralized Bone, No Fetuin

Calcium or Phosphate in Mineral, μmol

<table>
<thead>
<tr>
<th>Location of Mineral</th>
<th>Calcium</th>
<th>Phosphate</th>
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<td>Solution</td>
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Figure 4
Figure 6

Interval between mixing to achieve 5mM Calcium and Phosphate and Addition of Demineralized Bone

Calcium or Phosphate in Bone, μmol

0 10 24

0 2 4 6 8 10 12

1.5

Calcium

Phosphate
Figure 8
Rat Tail Tendon

Non Calcified

Calcified *in vitro*

2000 X

60,000 X

Elemental Analysis of the 60,000 X Field
Figure 10

Sephadex G-25 + Fetuin

Sephadex G-25, No Fetuin

Sephadex G-75 + Fetuin

Calcium

Phosphate

Calcium or Phosphate in Mineral, μmol

Location of Mineral

Sephadex G-25

Solution

Sephadex G-25

Solution

Sephadex G-75

Solution

* *