

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, whereas molecules larger than a 40-kDa protein are excluded. Based on these observations, we proposed a novel mechanism for fibril mineralization: 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 demonstrated that fetuin determines the location of mineral growth; in the presence of fetuin mineral grows exclusively within the fibril, whereas in its absence mineral grows in solution outside the fibril. Additional experiments showed 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 termed 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, that of a gatekeeper allowing 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 paradoxi-

cally 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 α 2-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). Because fetuin can trap only 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

[§]The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. A–D.

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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, whereas the serum fetuin concentration in the fetus and neonate is typically far higher (16). Fetuin is also one of the most abundant noncollagenous proteins found in bone (17–22), with a concentration of about 1 mg fetuin/g bone in rat (21), bovine (17), and human (19, 23) bone. Despite 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 because of the spontaneous formation of apatite crystals, the presence of added fetuin sustains elevated calcium levels for at least 24 h (24).

EXPERIMENTAL PROCEDURES

Materials—Male albino rats (Sprague-Dawley derived) were purchased from Harlan Laboratories. Alizarin red S, bovine fetuin, acrylamide, and bisacrylamide were purchased from Sigma, and Sephadex G25 and G75 were obtained from GE Healthcare.

Tibias were dissected from 22-day-old rats and cut to obtain a 1-cm 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 (30); the median diameter of the bone sand was 0.5 mm. Rat tibias and bovine bone sand were demineralized for 72 h at room temperature in 0.5 M 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 -20°C 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 rehydrated by overnight equilibration in ultra-pure water before use. Chondroitin sulfate A (bovine trachea), purchased from Calbiochem, was dialyzed extensively against 50 mM NH_4HCO_3 using a 100-kDa molecular weight cut-off dialysis membrane (Spectra/Por Biotech) and freeze-dried. Poly-L-glutamic acid (50–100 kDa) was obtained from Sigma. The University of California, San Diego 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 tem-

perature; 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 24 h 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).

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 homogeneous nucleation in the resulting unstable solution (24). One ml of 0.2 M HEPES, pH 7.4, containing 10 mM CaCl_2 was placed into one 10 \times 75-mm test tube, and another 1 ml of 0.2 M HEPES, pH 7.4, containing 10 mM sodium phosphate (also pH 7.4) was placed into a second tube. A disposable pipette was 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 5 mg of bovine fetuin/ml of buffer. Unless otherwise stated, the matrices tested using this procedure were added immediately after mixing to achieve the final 5 mM calcium and phosphate conditions; these included a 1-cm segment of hydrated, demineralized, tibia midshaft from a weanling rat (dry weight about 4 mg); hydrated, demineralized, bovine bone sand (4 mg dry weight); hydrated rat tail tendons (4 mg dry weight); hydrated Sephadex G25 or G75 (4 mg dry weight); and single 1 \times 5 \times 5-mm segments of 4 or 40% polyacrylamide slab gels (40% is 39.33 g of acrylamide and 0.67 g of bisacrylamide/100 ml). 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 s to sediment mineral. The supernatant was then diluted 1:4 with 0.2 M 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 50-ml volume of fetuin calcification solution (5 mM calcium and phosphate, 0.2 M HEPES, pH 7.4, 45 mM NaHCO_3 , 5 mg/ml fetuin, and 0.02% azide) and mixed end-over-end at room temperature for 2 days. For subsequent recalcification 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 yielded 4 mg of demineralized bone matrix.

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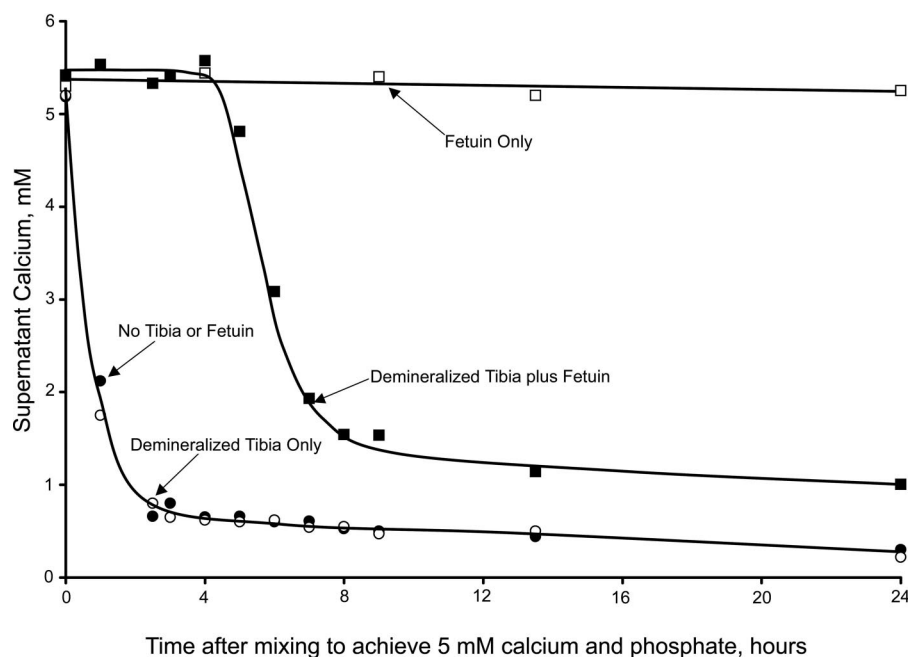


FIGURE 1. The recalcification of bone by using fetuin to selectively inhibit mineral growth outside the collagen fibril: time course of supernatant calcium. The test matrix was a 1-cm segment cut from the midshaft region of a rat tibia and demineralized in EDTA for 72 h (26). The solutions for the calcification test were prepared as described (24) and contained 2 ml HEPES, pH 7.4, with 5 mM 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 three replicate solutions.

For preparation of recalcified bone matrix for spectroscopic analysis, 4 mg of demineralized bovine bone sand (dry weight) was again added to each of three 50-ml volumes of fetuin calcification solution and mixed end-over-end at room temperature for 2 days. The recalcified bone sand was dried and ground in an agate mortar; an equivalent amount of non-demineralized bovine bone 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 Fourier transform infrared (FTIR)² spectrometer.

To prepare calcified tendon collagen for scanning electron microscopy, 4 mg of rat tail tendon (dry weight) was added to a 50-ml volume of fetuin calcification solution and mixed end-over-end at room temperature for 2 days. Samples of calcified and noncalcified 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.

RESULTS

Bone Can Be Recalcified by Using Fetuin to Selectively Inhibit Mineral Growth outside the Collagen Fibril—We first determined whether fetuin is able to selectively favor the recalcification 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 10 mM calcium to obtain a homogenous solution containing each ionic component at 5 mM in a pH 7.4 buffer. Previous studies have shown that a calcium phosphate mineral forms throughout this solution within minutes of mixing, whereas 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 was only sufficient calcium and phosphate to restore ~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 Fig. 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 Fig. 1, if both fetuin and a demineralized tibia are present, there is a decrease in solution calcium that begins about 5 h after the addition of the tibia, and solution calcium is reduced by about 4-fold at 8 h. Chemical analysis showed that the amount of calcium and phosphate incorporated into the tibia at 24 h 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 (Fig. 2). The recalcified tibias stained uniformly for calcification with Alizarin red, and von Kossa staining of tibia sections showed that calcification foci was 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 recalcification 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 5 mM calcium and phosphate, and solution calcium levels fell 5-fold within 2 h of mixing (Fig. 1). The presence of a demineralized tibia had no significant impact on the rate of calcium loss from solution in this experiment (Fig. 1). After a 24-h incubation of a demineralized tibia 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 (Fig. 2), and that 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 cal-

² The abbreviations used are: FTIR, Fourier transform infrared; BGP, bone Gla protein; MPG, matrix Gla protein.

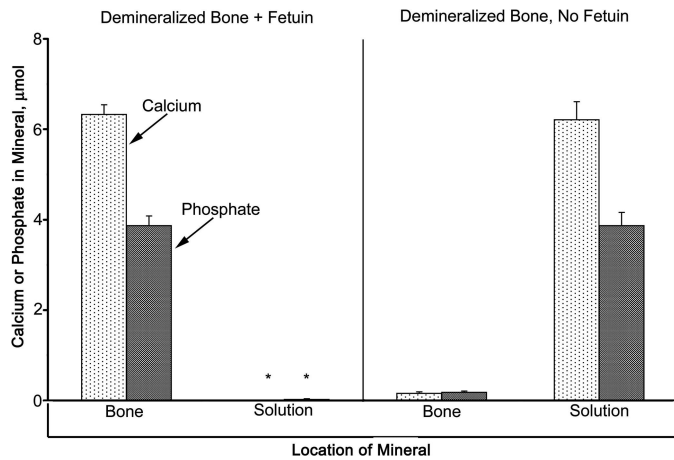


FIGURE 2. **The recalcification of bone by using fetuin to selectively inhibit mineral growth outside the collagen fibril: analysis for mineral calcium and phosphate.** The experiment described in the legend for Fig. 1 was terminated at 24 h, and the mineral that precipitated outside of the tibia was separated from the tibia. Then the mineral precipitate and tibia were both analyzed for calcium and phosphate. The results show the mean \pm S.D. of the measurements made on the three replicate bone samples at each condition.

cium and phosphate determines the location of the calcium phosphate mineral growth. In the absence of fetuin, mineral growth occurs primarily in the solution outside of bone collagen, whereas 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 recalcification procedure. Ground bone, rather than a tibia, was used for this test 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 recalcification solution was increased to 50 ml so that the calcium in the recalcification solution (250 μ mol) would exceed the calcium originally found in the bone matrix (114 μ mol). Finally, some of the samples were subjected to as many as three consecutive recalcification cycles, each in fresh 50-ml volumes of recalcification solution.

The first experiment examined the capacity of demineralized bone to take up mineral during three successive recalcification cycles. As seen in Fig. 3, the greatest increase in mineral occurred in the first recalcification cycle, and it 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 recalcification cycle does not significantly increase the mineral content of non-demineralized bone (Fig. 3). This observation shows that the incorporation of mineral into bone using this procedure requires prior demineralization.

Evidence That the Mineral in Recalcified 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 recalcified bone is

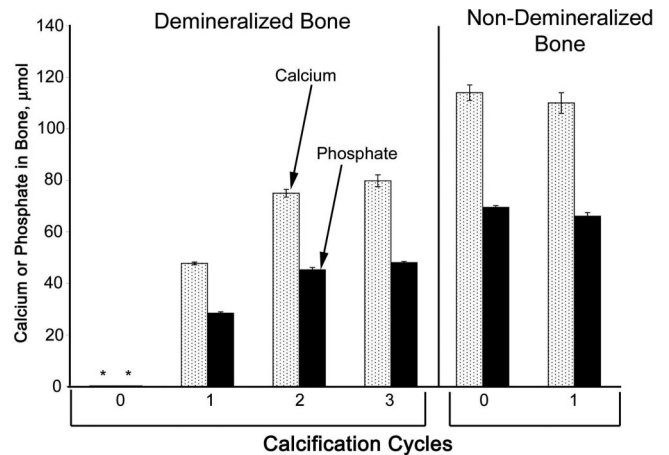


FIGURE 3. **Evidence that the capacity of bone collagen for mineral is limited.** Either 4 mg of demineralized bone sand or an amount of non-demineralized bone sand with the same collagen content (18 mg) was added to a 50-ml volume of 0.2 M HEPES, pH 7.4, containing 5 mg/ml fetuin, 5 mM calcium, and 5 mM phosphate, and the solution was mixed end-over-end at room temperature for 2 days. For subsequent recalcification 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 \pm S.D. of the measurements made on the three replicate bone samples at each condition.

similar to the mineral found in bone prior to demineralization. 1) The molar calcium to phosphate ratios calculated from the data presented in Fig. 3 range from 1.68 ± 0.03 for the first recalcification cycle to 1.66 ± 0.03 for the second and third cycles. These ratios are not significantly different from the ratios calculated from the Fig. 3 data for non-demineralized bone (1.66 ± 0.02 and 1.64 ± 0.03). 2) The powder x-ray diffraction spectrum obtained for demineralized bone after one recalcification cycle is comparable with the spectrum obtained for bone prior to demineralization (Fig. 4), and the diffraction peaks seen in both spectra are in the positions expected for synthetic hydroxyapatite crystals (30). 3) The FTIR absorbance spectra obtained for demineralized bone after one recalcification cycle is comparable with the spectrum obtained for bone prior to demineralization (Fig. 4). In the recalcified bone, the peak heights obtained for mineral components (phosphate and carbonate) are reduced relative to those for protein components (amide I and II); this observation is consistent with the fact that, after a single recalcification cycle, the partially remineralized bone has only about 40% of the mineral content of non-demineralized bone (Fig. 3).

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.

Fig. 5 shows that fetuin concentrations of 1–10 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.

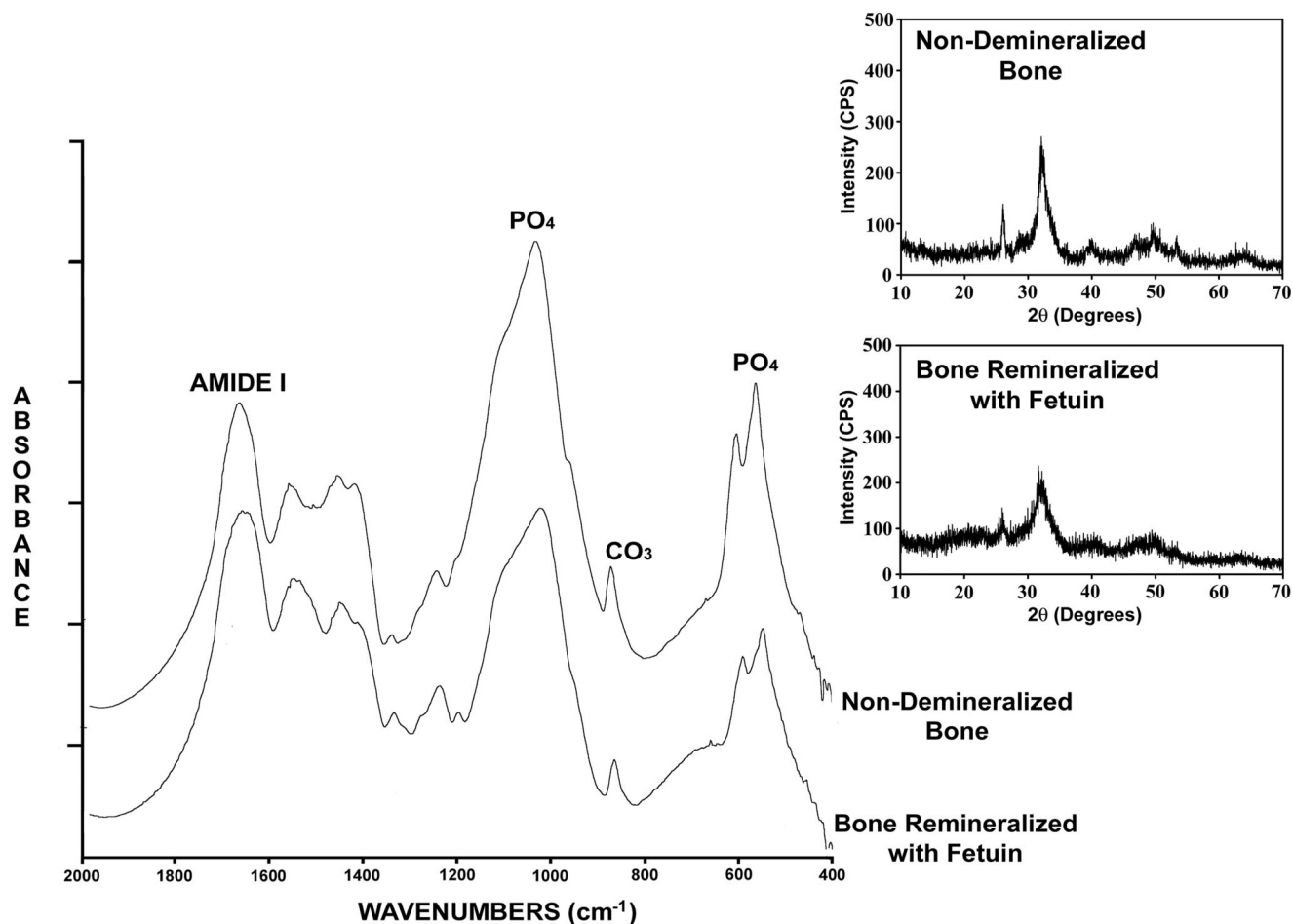


FIGURE 4. FTIR and powder x-ray diffraction spectra of bone that has been recalcified by using fetuin to selectively inhibit mineral growth outside the collagen fibril. Demineralized bovine bone sand was recalcified with fetuin as described in the legend for Fig. 3, and samples of the recalcified bone and of non-demineralized bone were each ground to a fine powder. The graph shows the FTIR spectrum of each sample, and the insets show the powder x-ray diffraction spectrum (see "Experimental Procedures" for details).

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 crossover between 0.25 and 0.1 mg/ml fetuin.

Because 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 seen in supplemental Fig. A, reducing the concentration of calcium and phosphate from 5 to 4 mM decreased the minimum amount of fetuin needed to achieve the selective calcification of collagen from 1 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, as the data in Fig. 1 show that fetuin maintains a high concentration of calcium for at least 24 h. To test this possibility, we examined the impact of delaying collagen addition on its calcification. As shown in Fig. 6, collagen is still efficiently calcified even when it is added 10 h after mixing to create the 5 mM calcium and phosphate. There was a significant reduction in calcium and phosphate incorpo-

ration when the collagen was added 24 h after mixing ($p < 0.01$; Fig. 6), and the total amount of mineral incorporated was reduced about 25%.

An experiment was carried out 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 Fig. B, chondroitin sulfate (molecular mass > 100 kDa) is unable to drive the selective calcification of collagen, whereas poly-L-glutamic acid (molecular mass > 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 because of a reduced ability to retard mineral growth in the solution outside the collagen fibril.

We had 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

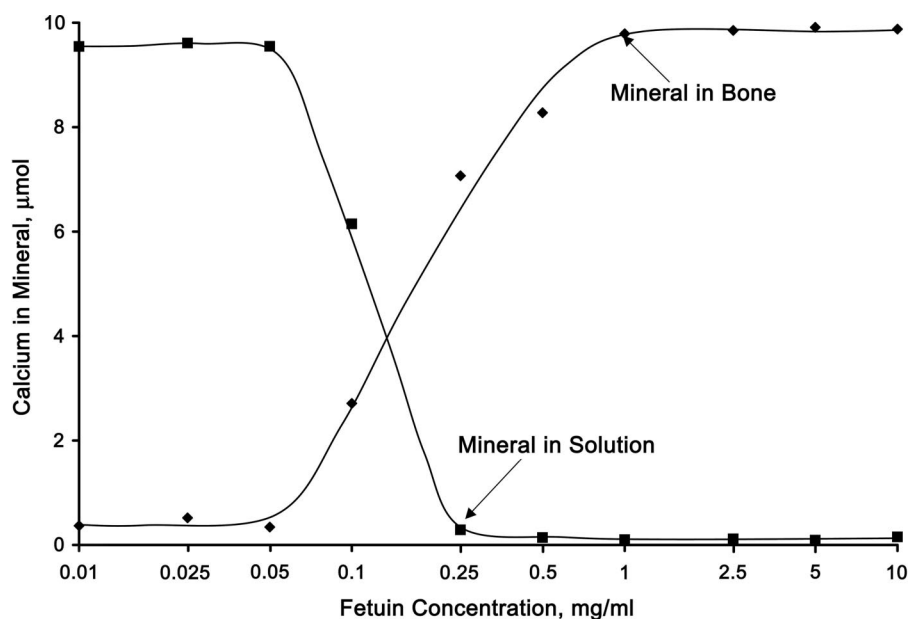


FIGURE 5. Dependence of bone collagen calcification on fetuin concentration when homogeneous crystal formation is driven by 5 mM calcium and phosphate. Four mg of demineralized bone sand was added to a 2-ml volume of 0.2 M HEPES, pH 7.4, containing 5 mM calcium, 5 mM phosphate, and the indicated concentration 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).

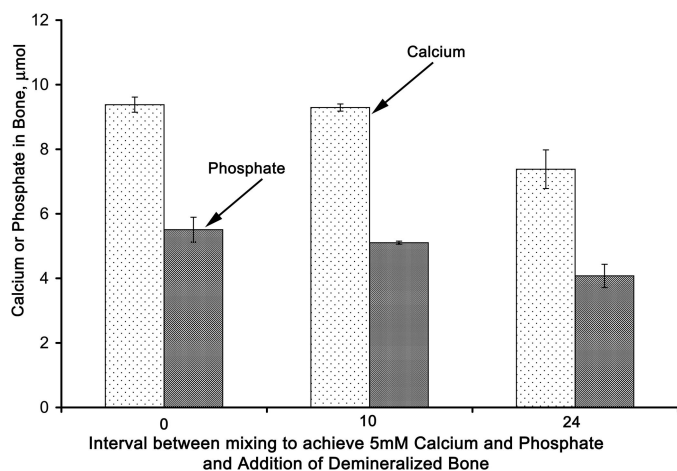


FIGURE 6. Evidence that fetuin sustains conditions that calcify bone collagen. Two-ml volumes of 0.2 M HEPES, pH 7.4, were prepared that contained 5 mM calcium, 5 mM phosphate, and 5 mg/ml fetuin. Four mg of demineralized bone sand was added at the indicated times after mixing calcium and phosphate. 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 \pm S.D. of the measurements made on the three replicate bone samples at each condition (see "Experimental Procedures" for details).

fibril (9). The results of this experiment show that BGP prevents mineral formation inside the collagen fibril (supplemental Fig. 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 was 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 ten-

don were added to calcification solutions identical to those used for the recalcification of demineralized tibias, and tendon calcification was evaluated using the same procedures. There was again a decrease in solution calcium that began 5 h after the addition of the tendons, and solution calcium was reduced 4-fold by 8 h (not shown). After 24 h, 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 (Fig. 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 Fig. 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 a 24-h incubation, chemical analysis showed that all mineral was in a precipitate outside of the tendon collagen, not within the collagen (Fig. 7), and the tendons did not stain with Alizarin red or von Kossa (supplemental Fig. 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 Fig. 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 (Fig. 8, bottom panels) demonstrated that calcium and phosphate co-localize with the collagen fibers. Electron dispersive x-ray spectra confirmed that calcified tendon collagen contained calcium and phosphate (supplemental Fig. D).

Synthetic Matrices That Have Size Exclusion Characteristics Similar to Type I Collagen Can Be Calcified by Using Fetuin to Selectively Inhibit Mineral Growth outside the Matrix—If the role of the type I 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 5 mM calcium and phosphate. Sephadex G25 was chosen for the first test, because 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

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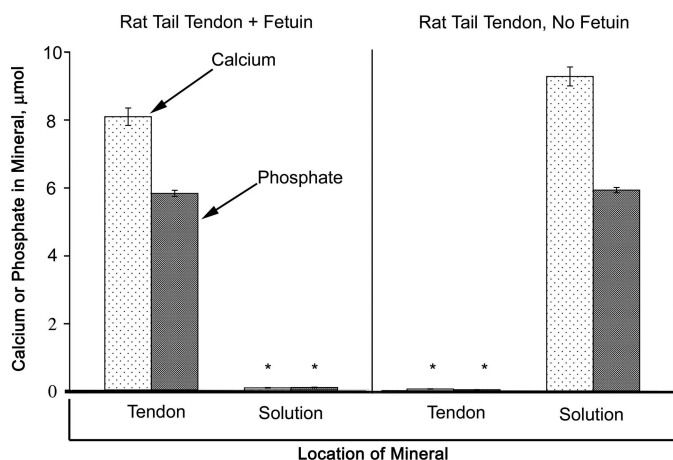


FIGURE 7. Calcification of tendon collagen by using fetuin to selectively inhibit mineral growth outside the collagen fibril: analysis for mineral calcium and phosphate. The solutions for the calcification test were prepared as described (24) and contained 2 ml of HEPES, pH 7.4, with 5 mM calcium and 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 24 h 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 \pm S.D. of the measurements made on the three tendon samples at each condition.

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 h after the addition of Sephadex G25, and solution calcium was reduced 5-fold by 8 h (Fig. 9). 2) Chemical analysis showed that the amount of calcium and phosphate found within Sephadex G25 at 24 h 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 (Fig. 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 showed 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 (Fig. 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 predicted that fetuin should be able to freely penetrate the interior of the gel bead (a result confirmed herein; 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-h period of observation (Fig. 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 (Fig. 10). 3) Alizarin red staining showed that none of the Sephadex G75 beads were calcified (not shown).

Rat Tail Tendon

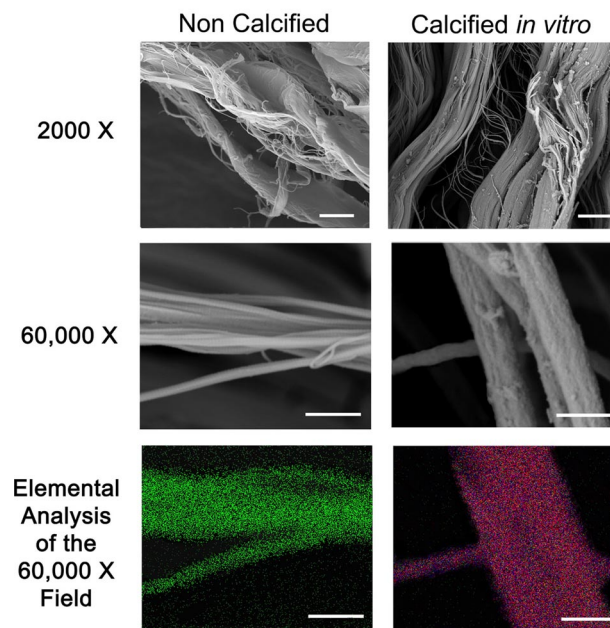


FIGURE 8. Scanning electron microscopy shows that mineral is located within the collagen fibers of tendon that has been calcified using fetuin. The procedure described in the legend for Fig. 3 was used to calcify 4 mg of rat tail tendon (dry weight). The calcified collagen was washed with 0.05% KOH, 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 $\times 60,000$ field immediately above: carbon is green; calcium is blue; phosphorus is red; and areas containing calcium and phosphorus are purple. (The electron dispersive x-ray spectra of these $\times 60,000$ fields are shown in supplemental Fig. C.) Bars are 20 μm for the top row of images and 1 μm for the middle and bottom rows (see "Experimental Procedures").

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, whereas 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 I 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 demonstrated that fetuin is all that is needed to determine the location of mineral growth; in the presence of fetuin, mineral grew within the collagen fibril, whereas in its absence mineral grew in the solution outside of collagen. The resulting calcification reaction was stunningly rapid and extensive; after incubation for just 8 h, the concentration of calcium in the tibia was more than 2000-

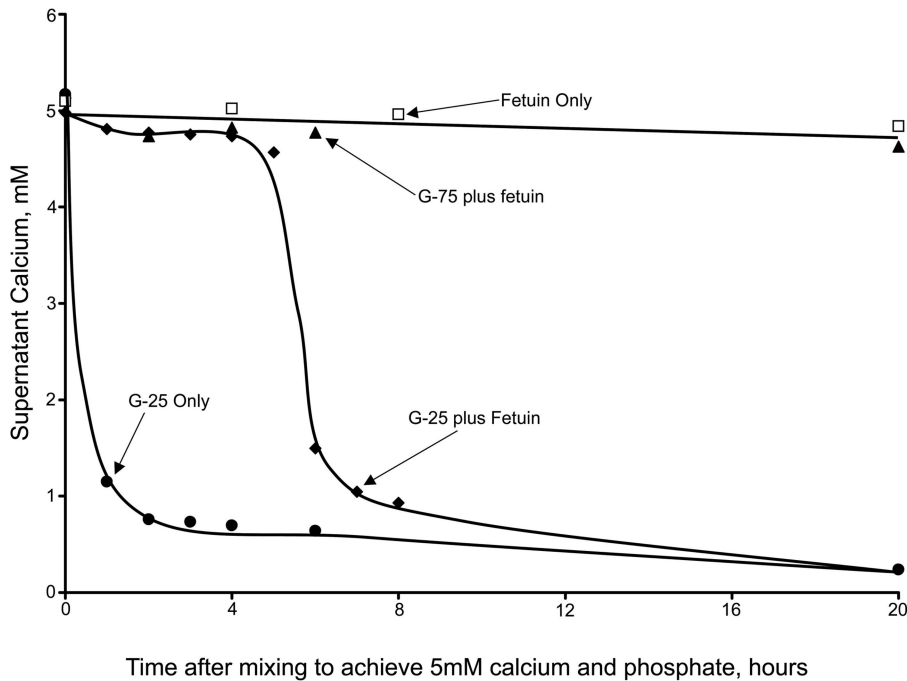


FIGURE 9. Calcification of Sephadex G25 by using fetuin to selectively inhibit mineral growth outside the gel beads: time course of supernatant calcium. The solutions prepared for the calcification test contained 2 ml of HEPES, pH 7.4, with 5 mM calcium and phosphate and fetuin only, Sephadex G25 only, fetuin plus Sephadex G25, and fetuin plus Sephadex G75. Each solution was placed into a 10 × 75-mm 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 three replicate solutions.

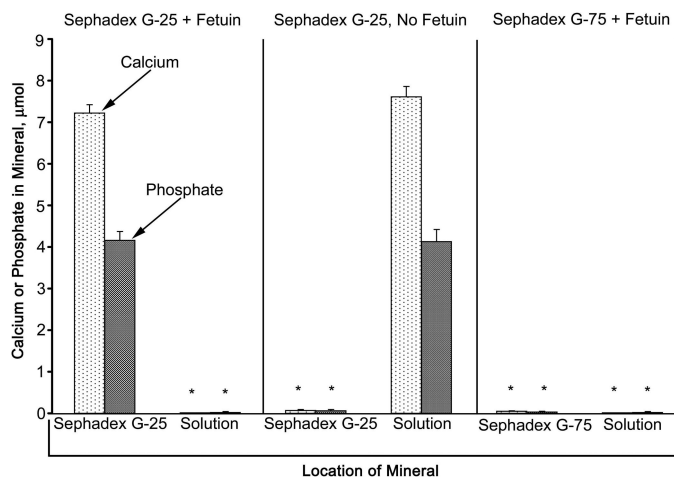


FIGURE 10. Calcification of Sephadex G25 by using fetuin to selectively inhibit mineral growth outside the gel beads: analysis for mineral calcium and phosphate. The experiment described in the legend for Fig. 7 was terminated at 24 h, the mineral that precipitated outside of the Sephadex was separated from the Sephadex using a 20- μ m sieve, and the mineral precipitate and Sephadex were both analyzed for calcium and phosphate. The results show the mean \pm S.D. of the measurements made on the three replicate Sephadex samples tested at each condition.

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 of \sim 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 with the amount of min-

eral 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 molar calcium-to-phosphate ratio, FTIR spectrum, and powder x-ray diffraction spectrum comparable with that of bone mineral. The same observations have been made using the chemically identical type I 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 I collagen fibril. We reasoned that if the role of the type I collagen fibril in this calcification mechanism is merely to provide an aqueous compartment that excludes fetuin but not calcium and phosphate, then 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 showed 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, whereas 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 suggested previously 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 μ g MGP/ml is sufficient to prevent the fetuin-dependent calcification of collagen.³ These *in vitro* experiments may explain why the overexpression of MGP in bone inhibits collagen calcification *in vivo* (35) and does not promote it.

³ F. A. Villa and P. A. Price, personal observations.

Mineralization by Inhibitor Exclusion

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 recalcification in order to stimulate local bone formation.

The ability to calcify purified type I collagen could also have uses. Metallic, plastic, and other noncollagenous 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 Is 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 the 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 predetermined 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 a 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 have demonstrated that fetuin is both necessary and sufficient for calcification of the type I collagen fibril.

We term this new calcification mechanism mineralization by inhibitor exclusion, the selective calcification of the type I 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 should 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 recalcified by these procedures with 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 90 days), 10 mg/ml at birth (gestational age 280 days), 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, as our present study shows that acceleration of mineral formation *in vitro* increases the amount of fetuin needed to support collagen calcification (Fig. 5 and supplemental Fig. A). 2) We have recently shown that higher serum fetuin levels are associated significantly with higher total hip, lumbar spine, and whole body bone mineral density 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 bone mineral density. 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 levels of calcium and phosphate (Fig. 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.

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