THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 7, pp. 4594–4604, February 13, 2009

Tissue-nonspecific Alkaline Phosphatase Is Required for the Calcification of Collagen in Serum

*A POSSIBLE MECHANISM FOR BIOMINERALIZATION**[S]

Received for publication, April 25, 2008, and in revised form, December 18, 2008 Published, JBC Papers in Press, December 19, 2008, DOI 10.1074/jbc.M803205200

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Previous studies have shown that the type I collagen of tendon and demineralized bone both calcify rapidly in serum. The speed, collagen matrix-type specificity, and extent of the re-calcification of demineralized bone in serum suggest that the serum calcification activity identified in these studies may participate in normal biomineralization. Because of its presence in serum and its long history of association with the normal mineralization of the collagen matrix of bone, tissue-nonspecific alkaline phosphatase (TNAP) is an obvious candidate for a protein that could be a component of serum calcification activity, and experiments were therefore carried out to test this possibility. These experiments show that the inactivation of TNAP in serum prevents collagen calcification, and that the addition of physiological levels of purified TNAP restores the ability of TNAP-deficient serum to calcify collagen. Additional experiments show that the role of TNAP in collagen calcification is to activate a serum nucleator of apatite crystal formation. Based on these and earlier studies, the mechanism of collagen calcification in serum requires at least four elements as follows. 1) A matrix (collagen fibrils) that is accessible to small apatite crystals but not large molecules (Toroian, D., Lim, J. E., and Price, P. A. (2007) *J. Biol. Chem.* 282, 22437–22447). 2) A large serum nucleator that generates small crystals, some of which diffuse into the fibrils. 3) A source of TNAP to activate the serum nucleator. 4) A large protein (fetuin) that selectively inhibits growth of crystals remaining in solution, thereby ensuring that only crystals within fibrils grow (Toroian, D. T., and Price, P. A. (2008) *Calcif. Tissue Int.* 82, 116–126).

Our goal is to understand the biochemical mechanism responsible for the calcification of collagen fibrils in normal bone formation. In the course of our investigations, we have discovered that purified type I collagen and demineralized bone matrix both calcify rapidly when incubated in serum in the absence of cells (1–4). The calcification of collagen is because of the presence of a serum calcification activity, one sufficiently potent that collagen calcifies when incubated in media containing as little as 1.5% serum but not in serum-free media alone (1–4). This serum calcification activity consists of one or more proteins that are 50–150 kDa in size (3, 4).

Although serum-driven collagen calcification is an *in vitro*, cell-free assay, there are several reasons to believe that it could be relevant to understanding mechanisms by which collagen fibrils are mineralized in nature. 1) The assay conditions are physiologically relevant; collagen added to serum calcifies when incubated at the temperature and pH of mammalian blood, without the need to add anything to serum to promote mineralization, such as β-glycerophosphate or phosphate (see Ref. 1 and references therein). 2) Serum is relevant to bone mineralization; osteoblasts form bone in a vascular compartment (5), and proteins in serum have direct access to the site of collagen fibril formation and mineralization, whereas proteins secreted by the osteoblast appear rapidly in serum. 3) Serum-driven calcification is evolutionarily conserved; the serum calcification activity appeared in animals at the time vertebrates acquired the ability to form calcium phosphate mineral structures, with no evidence for a similar activity in the serum of invertebrates (2). 4) Serum-driven calcification is specific; calcification is restricted to those structures that were calcified in bone prior to demineralization, with no evidence of calcification in cartilage at the bone ends or in cell debris (1, 3). 5) Serum-driven calcification can achieve the total re-calcification of demineralized bone;: serum-driven calcification progresses until the re-calcified bone is comparable with the original bone in mineral content and composition, radiographic density, and powder x-ray diffraction spectrum (3).

We have recently published two studies on the mechanism of serum-induced calcification of collagen fibrils (6, 7). The collagen fibril is about 70% water by volume (8), and during mineralization the water within the fibril is replaced by mineral (9–11). Our first study demonstrated that the water within the fibril is accessible to molecules and apatite crystals as large as a 6-kDa protein, and is inaccessible to molecules larger than a 40-kDa protein (7). Our second study showed that fetuin, a 48-kDa serum inhibitor of mineral growth (12, 13), determines the location in which the apatite crystals generated by the serum calcification activity can grow; in the absence of fetuin mineral grows in the solution outside collagen fibrils, whereas in the presence of fetuin mineral grows exclusively within collagen fibrils (6).

The results of these studies show that the mechanism of serum-induced calcification requires at least three elements as follows: 1) a matrix with an interior aqueous compartment that is accessible to small apatite crystals but not large molecules;

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table A and Figs. A–C.
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2) a large nucleator (not yet identified) that generates small crystals outside of the matrix, some of which diffuse into the matrix; and 3) a large protein (fetuin) that selectively inhibits the growth of those crystals remaining in solution outside the matrix, thereby ensuring that only crystals within the matrix grow. We have proposed the term "shotgun mineralization" for this calcification mechanism (6); crystals form throughout the solution, and only those that diffuse into a matrix grow.

Among the known serum proteins, tissue-nonspecific alkaline phosphatase (TNAP)[2] is arguably the most obvious candidate for a protein that could be a component of serum calcification activity. TNAP has a long and extensive history of association with normal bone calcification (14), and the molecular masses of the different glycosylated forms of the serum enzyme range from 126 to 141 kDa (15), which is within the molecular mass range expected for serum calcification activity (3). Serum TNAP consists of approximately equal amounts of the bone and liver isoforms of the enzyme. These isoforms have the same protein sequence but differ in *O*-linked glycosylation (22). Tissue-nonspecific alkaline phosphatase is a zinc metalloenzyme that hydrolyzes a broad range of phosphate monoesters, but its normal physiological substrate is presently unclear (14). As its name implies, tissue-nonspecific alkaline phosphatase is expressed in a wide variety of tissues, tissues that include bone, liver, and kidney (14). Despite its nonspecific expression in tissues, however, genetic deficiency in tissue-nonspecific alkaline phosphatase in mice and humans (called hypophosphatasia) is primarily associated with defects in bone mineralization (14, 16, 17). This observation, together with abundant expression of alkaline phosphatase at sites of bone mineralization (18, 19) in association with the outer surface of osteoblasts (20) and matrix vesicles (21), supports a probable role of the enzyme in normal bone mineralization.

The present experiments were carried out to determine whether the endogenous tissue-nonspecific alkaline phosphatase found in serum is a component of serum calcification activity. The results of these experiments show that TNAP is an essential component of serum calcification activity and that its role in the Shotgun Mechanism is to activate the serum nucleator of apatite crystal formation.

EXPERIMENTAL PROCEDURES

Materials—Bovine bone sand was prepared from the midshaft of bovine cortical bone as described (23). The bone sand was sized to obtain particles that pass through a 0.43-mm (35 mesh) sieve and are retained by a 0.25-mm (60 mesh) sieve, demineralized by extraction for 72 h with 0.5 M EDTA pH 7.5, and then washed exhaustively with water and dried. Tibias were dissected from newborn rats, demineralized in 0.5 M EDTA, pH 7.5, for 72 h at room temperature using a 300-fold molar excess of EDTA to mineral calcium, washed exhaustively with ultra pure water to remove all traces of EDTA, and stored at −20 °C until use (3). Rat tail tendons were prepared as described (3). Bovine kidney alkaline phosphatase (TNAP) with a specific activity of 1000 units/mg was obtained from Calzyme Laboratories (San Luis Obispo, CA); beryllium sulfate tetrahydrate was from Fluka; and (−)-tetramisole hydrochloride (levamisole), 1,10-phenanthroline monohydrate, purified bovine milk casein, purified type I collagen from bovine achilles tendon, and Alizarin red S were from Sigma. Newborn calf serum was purchased from Invitrogen and had a basal alkaline phosphatase activity of 0.2 unit/ml. A single batch of newborn calf serum was used for all experiments to eliminate serum as a possible variable. DMEM was prepared by supplementing each 500-ml volume of Dulbecco's modified Eagle's medium (Invitrogen) with 5 ml of penicillin/streptomycin (Invitrogen), 1 ml of 10% sodium azide, and 1.1 ml of 0.5 M sodium phosphate buffer, pH 7.4 (final medium P_i concentration = 2 mM).

Experiments with Neat Serum—In a typical re-calcification experiment, 4 mg of demineralized bovine bone sand (dry weight) was re-hydrated in water, placed in 2 ml of newborn calf serum in a 24-well plate (Costar 3524), and incubated in a humidified incubator at 37 °C and 7.5% CO_2 with continuous, gentle mixing using a plate shaker. In the calcification time course experiment (Fig. 1), three wells contained 4 mg of bone sand in 2 ml of newborn calf serum (NBCS); three wells contained bone sand in NBCS plus 10 units/ml TNAP; three wells contained bone sand in NBCS plus 30 mM levamisole; three wells contained bone sand in NBCS plus 30 μM beryllium sulfate; and three wells contained NBCS alone. At $t = 0$ and every 12 h thereafter, 25 μl were removed from every well, combined with 25 μl of 150 mM HCl, and analyzed for calcium; calcium uptake was determined from the decrease in serum calcium because of the presence of bone sand. To test the effect of added TNAP on calcification of different matrices (supplemental Fig. A), rat tail tendon segments and single demineralized newborn rat tibias were each separately incubated for 36 h in 2-ml volumes of NBCS and of NBCS containing 10 units/ml TNAP. To determine the effect of alkaline phosphatase inhibitors on bone re-calcification in serum, 4 mg of demineralized bone sand was incubated for 72 h in 2 ml of NBCS containing the indicated concentrations of beryllium (supplemental Fig. B), levamisole (supplemental Fig. C), and 1,10-phenanthroline (Fig. 2).

To separately assess the effects of alkaline phosphatase inhibitors on the initiation and growth of calcification in demineralized bone (Fig. 3), a 24-h preincubation in serum was used to introduce mineral foci within the collagenous matrix, and a subsequent incubation for 5 days in DMEM without serum was used to foster growth of the initial crystal nuclei, using procedures that have been described elsewhere (3). Alkaline phosphatase inhibitors were added at the serum initiation stage or at the DMEM growth stage.

Experiments with TNAP-deficient Serum in DMEM—Serum deficient in alkaline phosphatase activity was prepared using 1,10-phenanthroline, a zinc chelator that irreversibly inactivates the enzyme (24). In a typical experiment, 150 μl of 1 M phenanthroline in methanol was combined with 10 ml of newborn calf serum in a 15-ml conical tube, and the tube was loosely capped and placed for 72 h in a humidified incubator at 37 °C and 7.5% CO_2. The serum was then placed into dialysis tubing (molecular mass cut-off of 6–8 kDa) and dialyzed against four changes of 125 ml of DMEM each using the same

[2] The abbreviations used are: TNAP, tissue-nonspecific alkaline phosphatase or bone/liver/kidney alkaline phosphatase; DMEM, Dulbecco's modified Eagle's medium; NBCS, newborn calf serum.

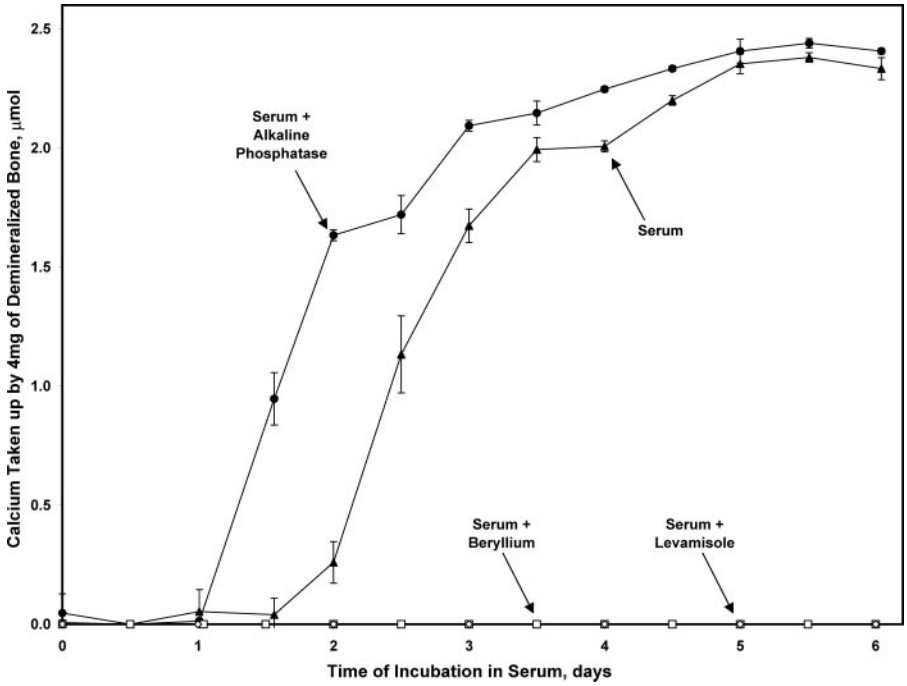

FIGURE 1. **Effect of alkaline phosphatase and of alkaline phosphatase inhibitors on bone re-calcification in serum, time course of calcium uptake.** Four-milligram samples of dry, demineralized bovine bone sand (0.25–0.43 mm) were re-hydrated in water and placed in wells of a 24-well plate (Costar 3524) containing 2-ml volumes of newborn calf serum alone, serum with 10 units/ml of TNAP, serum with 30 μM beryllium sulfate (a competitive inhibitor of TNAP), or serum with 30 mM levamisole (an uncompetitive inhibitor of TNAP); there were three wells per condition. Additional wells contained 2 ml of serum with no bone sand. The plate was then placed for 6 days in a humidified incubator at 7.5% CO_2 and 37 °C and mixed continuously with a plate shaker. Aliquots of 25 μl were removed from all wells every 12 h and analyzed for calcium. Calcium uptake was determined from the decrease in serum calcium because of the presence of bone sand; the *graph* shows the mean ± S.D. for the three wells in each treatment group (see "Experimental Procedures"). The difference in calcium uptake because of TNAP addition was highly significant ($p < 0.005$) for the 36–72-h time points.

incubator. The same dialysis procedure was also used to remove any non-protein associated zinc from the TNAP solution. Control serum was prepared by the same procedure using methanol vehicle alone. TNAP activity-deficient and control serum samples were frozen until use.

Experiments to examine the effect of TNAP depletion on the calcification of collagen matrices were carried out using 24-well cell culture clusters (Costar 3524, Corning Glass) in a humidified incubator at 37 °C and 5% CO_2. Each well contained 2 ml of DMEM with 2 mM P_i and 1% by volume of one of the following: control newborn calf serum; alkaline phosphatase-depleted serum; or alkaline phosphatase-depleted serum + 0.2 unit/ml of purified TNAP (see Table 3). The amount of matrix added to each 2-ml volume was as follows: a single hydrated, demineralized newborn rat tibia or a portion of purified type I collagen from bovine achilles tendon (3 mg dry weight; hydrated before use). Each tissue was then incubated for 6 days.

Experiments with Fetuin-deficient Serum in DMEM—Fetuin-depleted serum was prepared as described previously (6). In brief, a column packed with rabbit anti-bovine fetuin antibodies conjugated to Sepharose 4B was equilibrated with DMEM, and adult bovine serum was dialyzed against the same solution. The dialyzed bovine serum was then freed of fetuin by passing a 0.85-ml serum aliquot over the column at room temperature. The absorbance at 280 nm of each 1-ml fraction was determined; the four fractions with the highest absorbance were then pooled and diluted with DMEM until the absorbance equaled that of 10% bovine serum. Protein bound to the column was removed by washing the column with 100 mM glycine, pH 2.5. The effect of alkaline phosphatase and its inhibitors on apatite crystal formation in DMEM containing 10% fetuin-depleted serum was carried out using 24-well cell culture clusters in a humidified incubator at 37 °C and 5% CO_2 (see Ref. 6).

Biochemical Analyses—The procedures used for Alizarin red and von Kossa staining have been described (1). For quantitative assessment of mineral formation, matrices and precipitates were extracted for 24 h at room temperature with 1 ml of 0.15 M HCl. Calcium levels were determined colorimetrically using cresolphthalein complexone (JAS Diagnostics, Inc., Miami, FL), and phosphate levels were determined as described (25). Alkaline phosphatase activity was measured at pH 10.4 using *p*-nitrophenol phosphate as substrate (JAS Diagnostics).

RESULTS

Evidence That the Addition of Alkaline Phosphatase Accelerates the Rate of Collagen Calcification in Serum—TNAP is abundantly expressed at sites of bone mineralization (18, 19), where it is held in the external leaflet of osteoblast plasma membrane by a hydrophobic glycosylphosphatidylinositol anchor (32), and the local concentration of the enzyme near the osteoblast plasma membrane is likely to be far higher than the 0.2 unit/ml of alkaline phosphatase activity found in calf serum. As a first step in evaluating the effect of high local concentrations of TNAP on serum-induced collagen calcification, we investigated the impact of adding 10 units/ml of purified TNAP on the rate of bone re-calcification in new born calf serum at 37 °C and pH 7.4. Demineralized bovine bone sand 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. In the 2-ml serum volume used for this test, there is only sufficient calcium and phosphate to restore ~5% of the mineral that was present in the bone sand prior to demineralization.

Fig. 1 shows that the addition of purified bovine TNAP to serum markedly accelerates the rate at which serum calcium is taken up by bone in this experimental system, with half-maximal calcium uptake at 38 h in serum plus TNAP compared with 60 h in serum alone. The results of a repeat experiment were similar, with half-maximal calcium uptake at 40 h in serum plus added TNAP compared with 64 h in serum alone (data not shown). Although the addition of TNAP substantially accelerates the re-calcification of bone in serum, it does not significantly affect the amount of calcium taken up by bone after 6

TABLE 1

Effect of alkaline phosphatase and of alkaline phosphatase inhibitors on bone re-calcification in serum, analysis for calcium and phosphate

Bone sand was removed at the end of the 6-day experiment described in Fig. 1 and was stained with Alizarin red and then analyzed for calcium and phosphate. The table shows the mean and standard deviation for the calcium and phosphate incorporated into the three 4-mg bone sand samples in each treatment group.

Conditions	Bone sand re-calcification after 6 days at 37 °C		
	Intensity of Alizarin red staining	μmol of calcium in matrix	μmol of phosphate in matrix
Serum	+++	2.08 ± 0.02	1.06 ± 0.02
Serum + 10 units/ml alkaline phosphatase	+++	2.12 ± 0.05	1.10 ± 0.01
Serum + 30 mM levamisole	−	<0.1	<0.02
Serum + 30 μM beryllium	−	<0.1	<0.02

TABLE 2

Effect of alkaline phosphatase and of alkaline phosphatase inhibitors on serum phosphate levels after incubation for 9 days at 37 °C in the absence of bone matrix

One-ml aliquots of newborn bovine serum, serum with 10 units/ml of added tissue-nonspecific alkaline phosphatase, serum with 30 μM beryllium, or serum with 30 mM levamisole were incubated for 9 days at 37 °C in the absence of demineralized bone. The table shows the mean phosphate value obtained for the four measurements made on each serum sample; the standard deviation of the serum phosphate measurement was ±2%.

Conditions	Serum [P_i] before incubation	Serum [P_i] after 9 days at 37 °C
	mM	mM
Serum	2.46	2.52
Serum + 10 units/ml alkaline phosphatase	2.54	2.60
Serum + 30 mM levamisole	2.54	2.56
Serum + 30 μM beryllium	2.50	2.59

days of incubation (Fig. 1) or the total calcium and phosphate recovered from the re-calcified bone (Table 1). This result may reflect the effects of calcium and phosphate depletion during the 6-day incubation on the final extent of mineral formation in collagen, because most of the calcium and phosphate initially in the 2-ml serum aliquot has been deposited in bone by the 6-day time point. Previous studies have shown that the initial step in serum-induced apatite formation is highly dependent on the concentration of calcium and phosphate in solution, and will not continue to occur when the calcium phosphate ion product falls below about 2.5 mM[3] (1, 2).[4]

Fig. 1 also shows that the addition to serum of the alkaline phosphatase inhibitors beryllium (a competitive inhibitor (26)) and levamisole (an uncompetitive inhibitor (27, 28)) completely prevented the uptake of serum calcium by bone, and Table 1 shows that bone samples incubated in serum with either beryllium or levamisole had no detectable calcium or phosphate, and did not stain with Alizarin red.

A potentially trivial explanation for the effect of alkaline phosphatase on the kinetics of bone re-calcification in serum is that the enzyme could liberate sufficient phosphate from serum substrates to increase total ionic phosphate levels in serum and that this increase could accelerate the re-calcification of demineralized bone. To test this possibility, serum containing the 10 units/ml level of added TNAP was incubated for 9 days at 37 °C in the absence of bone matrix. As can be seen in Table 2, serum phosphate levels were not increased by the 9-day incubation with TNAP and were also not affected by the addition of alkaline phosphatase inhibitors. This experiment shows that any substrate hydrolysis that may occur during the 9-day incubation with alkaline phosphatase is not sufficient to produce a detectable increase in total ionic phosphate levels in serum.

An additional experiment was carried out to determine whether the addition of alkaline phosphatase accelerates the calcification of other collagenous matrices. This experiment used a 36- h incubation time, because the kinetic experiment (Fig. 1) showed that there is uptake of serum calcium by bone at this time only if alkaline phosphatase has been added to serum. As seen in supplemental Fig. A, rat tail tendons and demineralized newborn rat tibias stained positively for calcification with Alizarin red after incubation for 36 h in serum plus alkaline phosphatase, but not after incubation for 36 h in serum alone. Chemical analyses showed that there was a significant amount of calcium and phosphate in each matrix following the 36-h incubation in serum plus alkaline phosphatase, and there was no detectable calcium or phosphate in the same matrices after incubation for 36 h in serum alone (data not shown). These experiments demonstrate that alkaline phosphatase addition to serum has a comparable ability to accelerate the calcification of type I collagen matrices that were once calcified (demineralized tibias or bone sand) as well as collagenous matrices that were not previously mineralized (tail tendon).

Dose-dependent Effects of Alkaline Phosphatase Inhibitors on the Calcification of Collagen in Serum—The next experiments examined the dose-dependent effects of three different alkaline phosphate inhibitors on the calcification of collagen in serum. Fig. 2 shows that the re-calcification of demineralized bone in serum is dose-dependently inhibited by 1,10-phenanthroline, a zinc chelator that irreversibly inactivates alkaline phosphatase (24), with no evidence of Alizarin red staining or calcium and phosphate incorporation at the 1 and 3 mM doses of the inhibitor. Fig. 2 also shows that the endogenous alkaline phosphatase activity in serum is linked to the ability of serum to calcify collagen; the same dose of 1,10-phenanthroline that irreversibly inactivates TNAP activity in a serum aliquot also prevents bone re-calcification in the same serum aliquot.

The supplemental Fig. B shows that the re-calcification of bone in serum is dose-dependently inhibited by beryllium, with no evidence of Alizarin staining or calcium and phosphate incorporation at beryllium doses of 3 μM and above. Beryllium inhibition of TNAP is competitive, with a K_i = 0.45 μM (26), and in standard TNAP assays 50% inhibition of TNAP activity is consistently observed at 1 μM beryllium (29, 30). The fact that comparable 1 μM doses of beryllium cause 50% inhibition of bone re-calcification in serum (supplemental Fig. B) suggests that the ability of beryllium to inhibit serum-induced calcification is because of its ability to inhibit TNAP.[3] Although a number of mammalian enzymes have been tested for sensitivity to inhibition by beryllium (30), to our knowledge there are no

[3] It is of interest to note that the chronic beryllium administration to rats causes a failure of growth plate cartilage calcification that resembles rickets but is independent of vitamin D deficiency (29, 39). The original rationale for testing the effects of beryllium on TNAP was to better understand the molecular basis for beryllium-induced rickets (29, 39).

[4] P. A. Price, D. Toroian, and W. S. Chan, personal observations.

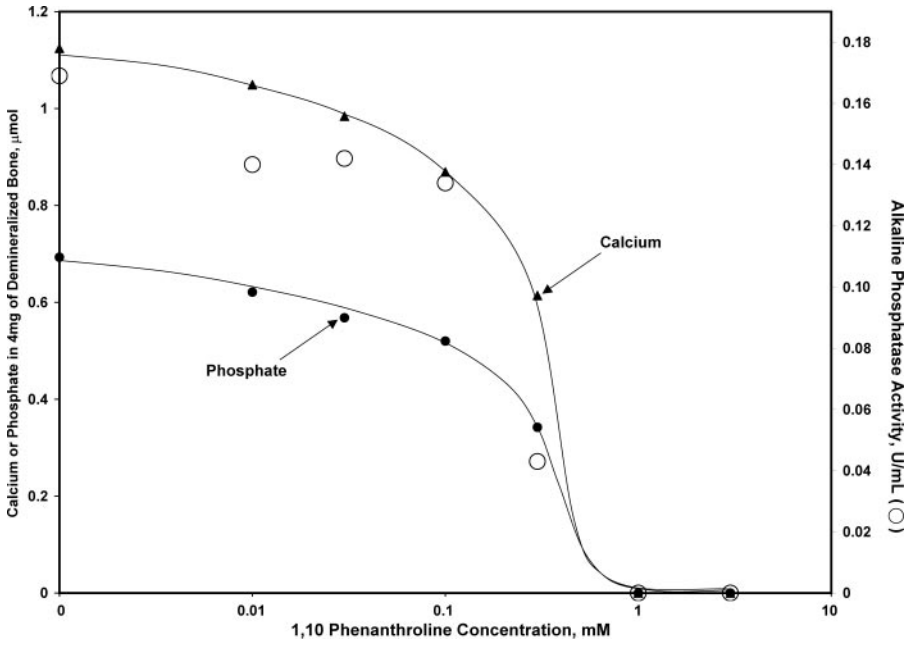

FIGURE 2. **Dose-dependent inhibition of bone re-calcification in serum by the irreversible alkaline phosphatase inhibitor 1,10-phenanthroline.** Four-milligram samples of dry, demineralized calf bone sand were re-hydrated in water and incubated for 72 h at 37 °C in 2 -ml aliquots of newborn calf serum containing the indicated concentrations of the zinc chelator 1,10-phenanthroline, an irreversible TNAP inhibitor. Each bone sample was first stained with Alizarin red and then analyzed for calcium and phosphate (see "Experimental Procedures"); the alkaline phosphatase activity remaining in serum was also measured at each 1,10-phenanthroline dose. The *graph* shows the calcium and phosphate incorporated into bone sand at each 1,10-phenanthroline dose and the alkaline phosphatase activity remaining in serum after the 72-h incubation (○). As can be seen, the 1 and 3 mM doses of 1,10-phenanthroline completely abolished alkaline phosphatase activity and prevented uptake of calcium and phosphate; these doses also completely prevented Alizarin red staining (data not shown), although lower doses did not.

mammalian serum enzymes other than alkaline phosphatase that are inhibited by micromolar beryllium concentrations.

The supplemental Fig. C shows that bone re-calcification is dose-dependently inhibited by levamisole, an uncompetitive alkaline phosphatase inhibitor (27, 28). There is 50% inhibition of bone re-calcification in serum containing 1 mM levamisole and complete inhibition of bone re-calcification at 10 and 30 mM levamisole. Previous studies have shown that comparable concentrations of levamisole are required for inhibition of TNAP activity in the epiphyseal plate cartilage of rachitic rats (31).

An additional experiment was carried out to verify that alkaline phosphatase inhibitors prevent the initiation of bone re-calcification, not the subsequent growth of the initial mineral nuclei. The design of this experiment is based on a previous study that showed that incubation of demineralized bone in serum for 24 h is sufficient to cause the treated bone to completely re-calcify during subsequent incubation in DMEM, but it is not sufficient for Alizarin red staining or detectable levels of calcium and phosphate incorporation (3). The effects of inhibitors on the serum-dependent formation of crystal nuclei within the matrix could therefore be evaluated by adding the inhibitors only to serum during the first 24 h of incubation, whereas the effects of the inhibitors on the subsequent growth of these nuclei could be assessed by adding the inhibitors only to DMEM during the 5 days of incubation. Fig. 3 shows that each of the three alkaline phosphatase inhibitors tested inhibited the initiation of bone calcification during the first 24 h in serum, but did not inhibit the growth of the initial mineral nuclei during the subsequent 5-day incubation in DMEM. Alkaline phosphatase inhibitors therefore prevent the serum-dependent initiation of bone re-calcification, not the subsequent growth of the initial mineral nuclei.

Evidence That Serum Calcification Activity Is Inhibited by TNAP Depletion and Restored by the Addition of Physiological Levels of TNAP—We next devised a method to prepare TNAP-depleted serum to further explore the role of TNAP in the serum-induced calcification of collagen. In this procedure, the endogenous alkaline phosphatase activity of serum is first irreversibly inactivated by treatment with the zinc chelator 1,10-phenanthroline, and the zinc-phenanthroline complex is then removed by dialysis against DMEM (which does not contain zinc). The resulting TNAP-depleted serum proved to have less than 1% of the initial alkaline phosphatase activity (Table 3) and is no longer able to induce the calcification of purified type 1 collagen (Fig. 4) or of demineralized newborn rat tibia (Fig. 5).

The alkaline phosphatase activity of newborn calf serum is 0.2 unit/ml. To confirm the essential role of TNAP in the serum-induced calcification of collagen, we therefore added 0.2 unit/ml purified TNAP to TNAP-deficient serum (Table 3). This addition restored the ability of serum to induce the calcification of purified type 1 collagen (Fig. 4) and demineralized newborn rat tibias (Fig. 5).

A control experiment was carried out to address the possibility that there might be non-protein-associated zinc in the TNAP solution, that this available zinc might activate a zinc-dependent protein in serum other than TNAP, and that the activation of this protein might actually be responsible for the ability of the TNAP solution to restore the calcification activity of phenanthroline-treated serum. The TNAP solution was first dialyzed exhaustively against DMEM using a 6–8-kDa molecular mass cut-off membrane to remove any non-protein-associated zinc; this dialysis step did not significantly diminish TNAP activity. In a repeat of the experiment shown in Fig. 4, we added 0.2 unit/ml of the dialyzed TNAP to TNAP-deficient serum. The results of this experiment were comparable with those shown in Fig. 4; the addition of dialyzed TNAP fully restored the ability of serum to induce the calcification of purified type 1 collagen (data not shown). Taken together, these observations provide convincing evidence that TNAP plays an essential role in the serum-induced calcification of collagen.

Effect of Alkaline Phosphatase and Its Inhibitors on the Formation of Apatite Crystals in Fetuin-deficient Serum That Con-

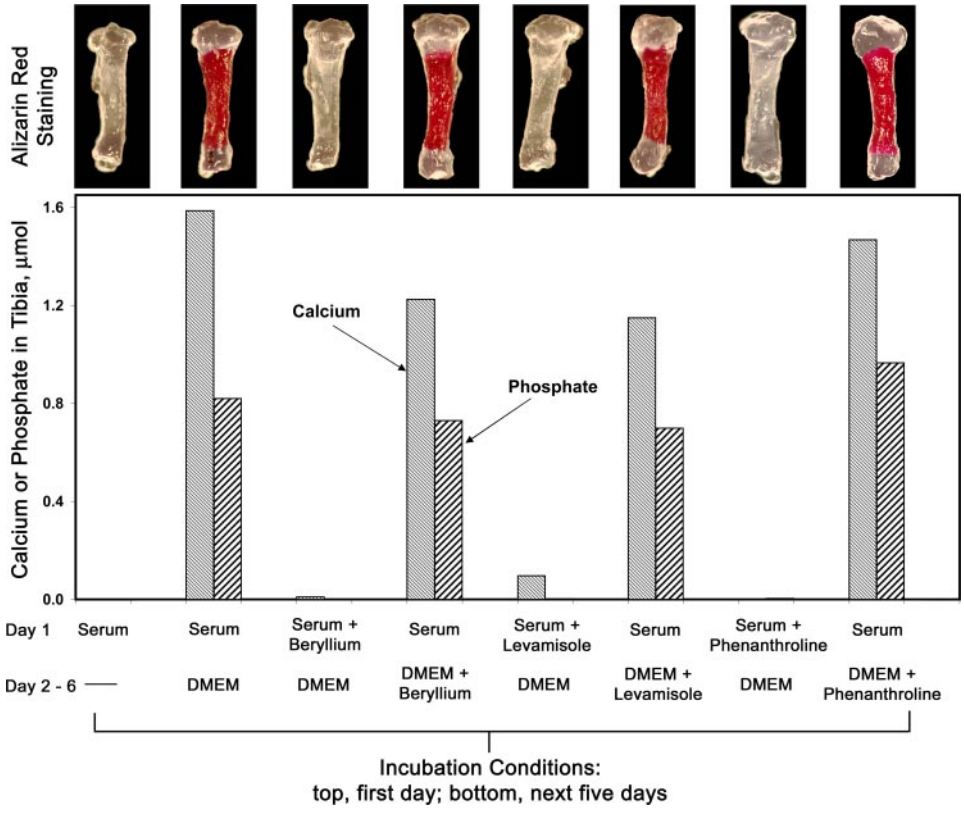

FIGURE 3. **Evidence that alkaline phosphatase inhibitors prevent initiation of bone re-calcification by serum but do not prevent subsequent crystal growth.** Demineralized newborn rat tibias were separately incubated for 24 h in 2-ml aliquots of newborn calf serum, or serum containing either 10 μM beryllium sulfate, 10 mM levamisole, or 1 mM 1,10-phenanthroline. Two of the tibias incubated in serum alone were removed and stained with Alizarin red. All other tibias were removed from serum, rinsed twice with 10 ml of DMEM, and then incubated for 5 days in 10 ml of DMEM or 10 ml of DMEM containing either 10 μM beryllium sulfate, 10 mM levamisole, or 1 mM 1,10-phenanthroline. Tibias were stained with Alizarin red and then analyzed for calcium and phosphate.

TABLE 3

The alkaline phosphatase activity in the serum samples used in these studies

The activity of alkaline phosphatase was determined by colorimetric assay in each of the newborn calf serum samples employed in this study as follows: control serum, alkaline phosphatase-depleted serum, and alkaline phosphatase-depleted serum supplemented with 0.2 unit/ml purified TNAP. Each sample was assayed in triplicate. See under "Experimental Procedures" for details.

Sample	Alkaline phosphatase activity
	units/ml
Control serum	0.18 ± 0.03
Alkaline phosphatase-depleted serum	<0.002
Alkaline phosphatase-depleted serum + 0.2 unit/ml purified TNAP	0.22 ± 0.03

tains No Collagen—In the course of this study, we discovered 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 (6). We further found that a massive mineral precipitate forms during the incubation of fetuin-depleted serum but not during the incubation of serum-containing fetuin (6). This mineral precipitate forms whether or not a collagen matrix is present. 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 (6). Because 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. When fetuin is removed from serum, the same number of mineral nuclei still form, and some of these no doubt still penetrate the fibril. All crystal nuclei are now free to grow, however. Because the vast majority of the nuclei are in the solution outside of the fiber, the only mineral formed in amounts that can be detected is the mineral precipitate found on the bottom of the well and not mineral within the fibril (6).

In this study, we have used fetuin-depleted serum to directly evaluate the possible role of TNAP in the formation of apatite crystals in serum that contains no collagen matrix. Fetuin was again removed from bovine serum using rabbit anti-bovine fetuin antibodies; the fetuin content of the resulting fetuin-depleted serum was over 1000-fold lower than the fetuin content of the original bovine serum (supplemental Table A). Fig. 6 shows that the addition of TNAP more than doubled the rate at which calcium is incorporated into crystals during incubation of DMEM containing 10% fetuin-depleted serum at 37 °C, with half-maximal calcium incorporation at 15 h in fetuin-depleted serum plus TNAP compared with 38 h in fetuin-depleted serum alone. The addition of TNAP also reduced the incubation time needed for the first visible evidence of crystal formation in this experiment (cloudiness), from 30 h in the absence of TNAP to 12 h in the presence. In agreement with previous studies (6), a massive precipitate could be seen in all wells by 48 h, and chemical analysis of this precipitate again confirmed that it is a calcium phosphate mineral (data not shown).

It is of interest to note that calcium is incorporated into mineral at a significantly faster rate in fetuin-deplete serum ($t_{1/2}$ = 38 h; Fig. 6) than in fetuin-replete serum ($t_{1/2}$ = 60 h; Fig. 1). Although the addition of TNAP accelerates calcium incorporation into mineral in both solutions, the rate of calcium incorporation into mineral in fetuin-deplete serum plus TNAP ($t_{1/2}$ = 15 h; Fig. 6) remains substantially faster than in fetuin-replete serum plus TNAP ($t_{1/2}$ = 38 h; Fig. 1). It seems probable that the relatively faster growth of mineral in fetuin-deplete serum is in part because of the fact that the crystals grow in the solution, whereas the mineral in fetuin-replete serum must grow within the collagen fibrils of the decalcified bone. The constraints imposed by the location of crystals within the collagen fibrils

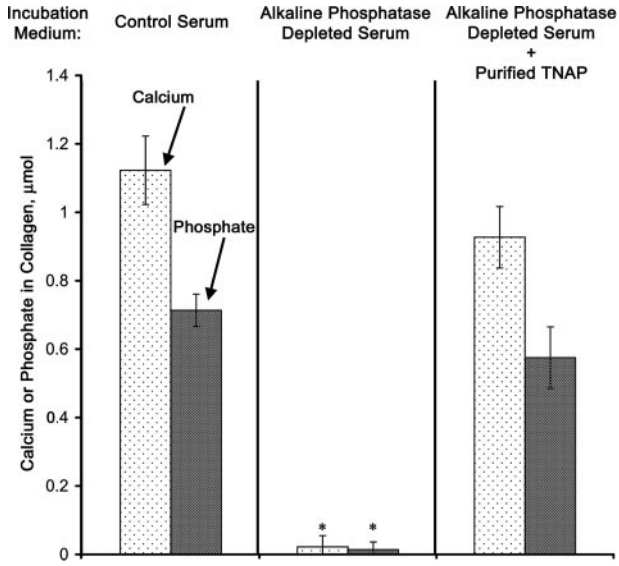

FIGURE 4. **Evidence that the calcification of type 1 collagen in serum is inhibited by TNAP depletion and restored by the addition of physiological levels of TNAP.** To further assess the role of TNAP in the serum-induced calcification of collagen, 3-mg amounts of purified bovine type I collagen were hydrated and separately incubated for 6 days at 37 °C in 2 ml of DMEM containing 2 mM P_i and 1% by volume of one of the following: control newborn calf serum; alkaline phosphatase-depleted serum; or alkaline phosphatase-depleted serum + 0.2 units/ml purified TNAP (see Table 3 for the alkaline phosphatase activity in each serum sample). This experiment was performed in triplicate. The data show the average calcium and phosphate in the purified collagen from each well; the *error bars* show the standard deviations. * = calcium or P_i in the extract is less than 0.01 μmol.

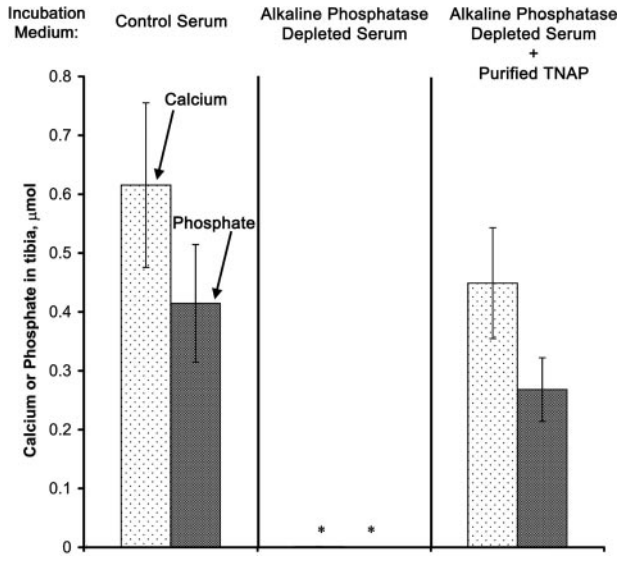

FIGURE 5. **Evidence that the re-calcification of bone in serum is inhibited by TNAP depletion and restored by the addition of physiological levels of TNAP.** Demineralized newborn rat tibias were separately incubated for 6 days at 37 °C in 2 ml of DMEM containing 2 mM P_i and 1% by volume of one of the following: control newborn calf serum; alkaline phosphatase-depleted serum; or alkaline phosphatase-depleted serum + 0.2 units/ml purified TNAP (see Table 3). This experiment was performed in triplicate. The data show the average calcium and phosphate in the purified collagen from each well; the *error bars* show the standard deviations. * = calcium or P_i in the extract is less than 0.01 μmol.

are likely to substantially reduce the overall growth of mineral in the case of fetuin-replete serum.

It is also of interest that the addition of purified TNAP to serum does not produce a proportional 50-fold increase in the

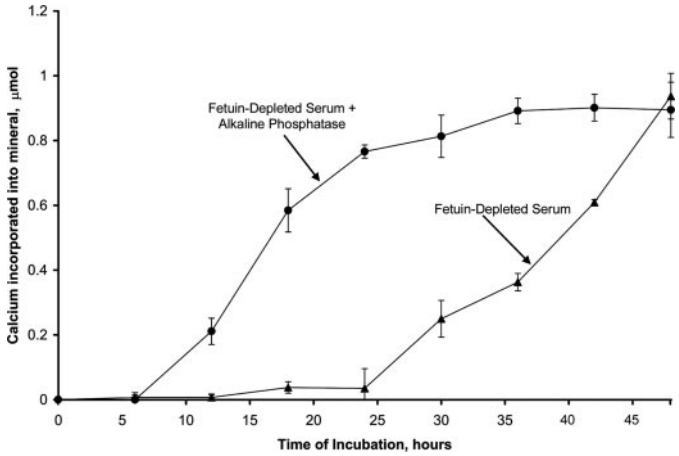

FIGURE 6. **Effect of alkaline phosphatase on the formation of apatite crystals in fetuin-depleted serum that contains no collagen, time course of calcium incorporation into mineral.** Solutions were prepared that contained DMEM with 2 mM P_i and either 10% fetuin-depleted bovine serum or 10% fetuin-depleted bovine serum plus 10 units/ml TNAP. One-ml volumes of each solution were placed into 3 wells of a 24-well plate and incubated for 2 days at 37 °C. Aliquots of 50 μl were removed from all wells every 6 h and analyzed for calcium. Calcium incorporated into mineral was determined from the decrease in solution calcium; the *graph* shows the mean ± S.D. for the three wells in each treatment group (see "Experimental Procedures").

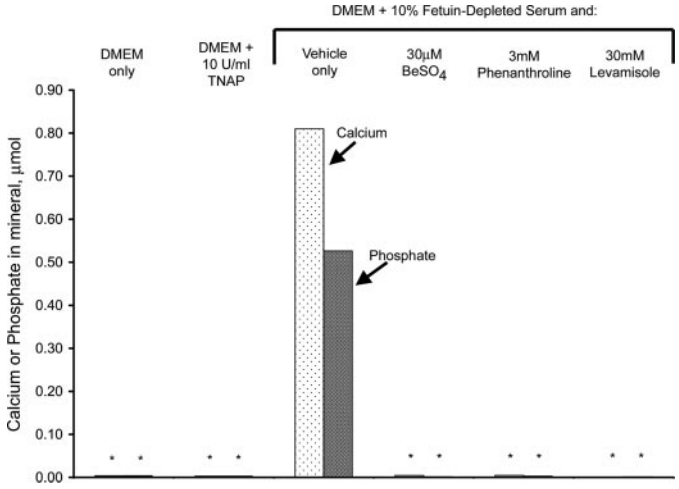

FIGURE 7. **Effect of alkaline phosphatase inhibitors on the formation of apatite crystals in fetuin-depleted serum that contains no collagen.** Solutions were prepared that contained DMEM with 2 mM P_i and the following: no additive; 10 units/ml TNAP; 10% fetuin-depleted bovine serum; 10% fetuin-depleted bovine serum plus 30 μM beryllium sulfate; 10% fetuin-depleted bovine serum plus 30 mM levamisole; and 10% fetuin-depleted bovine serum plus 3 mM 1,10-phenanthroline. One-ml volumes were placed into wells of a 24-well plate and incubated for 6 days at 37 °C. The media and any precipitate were removed from the well and centrifuged to pellet any precipitate, and the pellet fraction was analyzed for calcium and phosphate (see "Experimental Procedures" for details). The data show the calcium and phosphate in the pellet fraction. * = calcium or P_i in the extract is less than 0.01 μmol. A massive mineral precipitate was visible in the well containing DMEM plus 10% fetuin-depleted serum, although no precipitate could be detected in the other wells.

rate of collagen calcification in fetuin-replete serum (Fig. 1) or in the rate of apatite crystal formation in fetuin-deficient serum (Fig. 6). This observation indicates that there are other steps in the serum-induced apatite crystal formation pathway that become rate-limiting at high TNAP doses.

An additional experiment was carried out to further examine the effects of TNAP and its inhibitors on the formation of apatite crystals in fetuin-depleted serum. As seen in Fig. 7, the

formation of a mineral phase in DMEM containing 10% fetuin-depleted serum was completely prevented by 30 μM beryllium sulfate, 30 mM levamisole, or 3 mM 1,10-phenanthroline. This complete inhibition is remarkable, as the incubation was for 6 days although only 2 days are needed for mineral formation in the absence of inhibitor (Fig. 6). Fig. 7 also shows that the addition of TNAP to DMEM is not sufficient for the formation of a mineral phase in the absence of fetuin-depleted serum. We were also unable to achieve the calcification of a type 1 collagen matrix using DMEM plus TNAP, DMEM plus fetuin, or DMEM plus TNAP and fetuin (data not shown). These observations demonstrate that crystal generation requires a serum component other than TNAP or fetuin.

DISCUSSION

The present experiments were carried out to determine whether tissue-nonspecific alkaline phosphatase is a component of the serum activity that calcifies type I collagen. The results of these tests show that TNAP plays a critical role in the calcification of type I collagen in serum and that this role is to activate a serum nucleator of apatite crystal formation. Because of the potential importance of these results to an understanding of normal biomineralization, it is important to begin with a discussion of the experimental evidence.

The first experiments showed that the addition of purified alkaline phosphatase to serum consistently accelerates the calcification of demineralized bone, a collagenous matrix that was once calcified, and rat tail tendon collagen, a collagenous tissue that does not normally calcify *in vivo*. These observations show that the addition of alkaline phosphatase does not accelerate the calcification of matrices by virtue of its action on a matrix component that is unique to tissues that normally calcify, and suggest that TNAP may instead accelerate matrix calcification by acting on a component of serum that drives mineral formation.

TNAP is a phosphomonoesterase, and a potentially trivial explanation for the ability of alkaline phosphatase to accelerate collagen calcification in serum is that the enzyme could liberate sufficient phosphate from serum phosphomonoesterase substrates to increase total ionic phosphate levels, and that this increase in serum phosphate could accelerate the re-calcification of demineralized bone. This possibility was tested by measuring ionic phosphate levels in serum before TNAP addition, and after addition of TNAP and incubation for 9 days at 37 °C, and no significant increase in phosphate was observed (Table 2). We conclude that added TNAP does not increase the rate of collagen calcification by raising serum phosphate levels.

The next experiments showed that three inhibitors of tissue nonspecific alkaline phosphatase each prevent the calcification of collagen in serum. In each case, the inhibitor dose required for inhibition of serum-induced calcification is comparable with the dose previously found to inhibit purified TNAP *in vitro*. For the irreversible inhibitor 1,10-phenanthroline, there was also an excellent correlation between the inhibition of alkaline phosphatase activity in a serum aliquot and the reduction in serum-induced collagen calcification in the same aliquot (Fig. 2).

In a final test, the alkaline phosphatase activity of serum was inactivated by treatment with the zinc chelator 1,10-phenanthroline, and the zinc-phenanthroline complex was then removed by dialysis (Table 3). As anticipated, the resulting TNAP activity-deficient serum was unable to calcify either purified type 1 collagen (Fig. 4) or demineralized bone (Fig. 5). However, if the normal physiological level of purified TNAP was added to this TNAP-deficient serum (Table 3), the ability to calcify collagen was completely restored. These observations demonstrate that phenanthroline prevents the calcification of collagen in serum by virtue of its ability to inactivate TNAP, and these observations show that the addition of physiological levels of purified TNAP to serum is sufficient to restore the ability of TNAP-deficient serum to calcify collagen.

Evidence That Tissue Nonspecific Alkaline Phosphatase Is Required for the Formation of Apatite Crystals in Fetuin-deficient Serum—In the course of these studies, we discovered that removing fetuin from serum causes the formation of numerous apatite-like crystals; these crystals form whether or not a collagen matrix is present (6). We therefore carried out additional studies to determine the effect of alkaline phosphatase and its inhibitors on the formation of apatite crystals in fetuin-deficient serum. These experiments showed that adding purified bovine TNAP to fetuin-deficient serum approximately doubled the rate of crystal formation (Fig. 6). Doses of TNAP inhibitors that prevent the re-calcification of demineralized bone also prevented the formation of crystals in fetuin-deficient serum (Fig. 7). Because TNAP did not cause crystals to form in the absence of fetuin-depleted serum, TNAP must act on a serum component (the serum nucleator) to induce crystal formation.

Molecular Mechanism for the Calcification of Collagen Matrices—A critical problem in biomineralization is to understand how a matrix is mineralized. With this study, we have now partially solved this problem for the calcification of the type 1 collagen fibril in neat serum (6, 7). To our knowledge, these are the first studies that provide a defined molecular explanation for matrix mineralization.

The molecular mechanism that selectively calcifies collagen fibrils in serum requires the following four critical elements (Fig. 8). 1) A matrix with an interior aqueous compartment that is accessible to small apatite crystals but not large molecules is required. The collagen fibril is 70% water by volume (8), and during calcification the water within the fibril is replaced by mineral (10, 11). We have recently discovered that all of the water within the uncalcified fibril is in rapid diffusion equilibrium with molecules up to the size of a 6-kDa protein, and is completely inaccessible to molecules larger than a 40-kDa protein (7). Hydroxyapatite crystals up to the size of a 6-kDa protein (about 12-unit cells (7)) are therefore in principle able to attain rapid diffusion equilibrium with the entire volume of water within the collagen fibril, whereas large inhibitors of crystal growth are completely excluded.

2) A large nucleator (not yet identified) that generates small crystals outside of the matrix, some of which diffuse into the matrix, is required. The evidence for this key concept is compelling. In the absence of fetuin, massive numbers of crystals appear in serum within 2 days of incubation at 37 °C. These crystals have the same XRD spectrum as the apatite-like crystals found in bone (6), and the crystals form in the solution whether or not a collagen matrix is present. Although the iden-

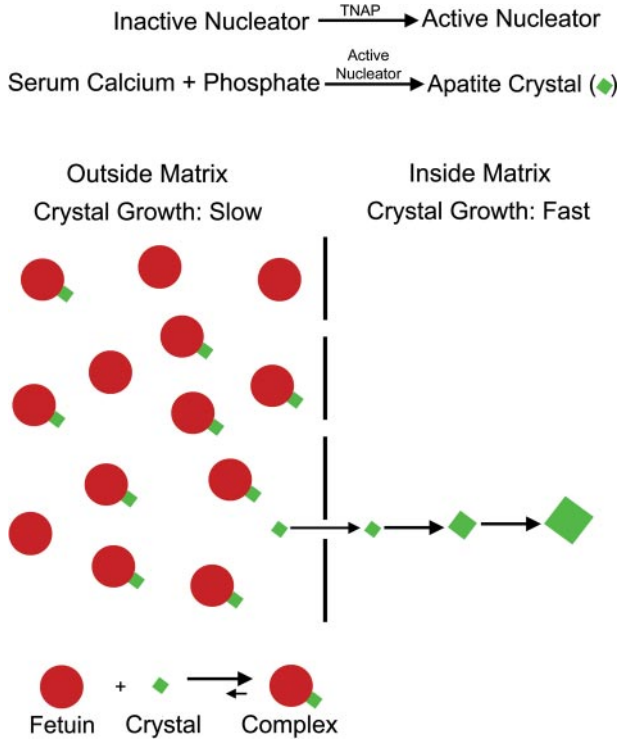

FIGURE 8. **The shotgun mineralization mechanism.** TNAP activates the high molecular weight nucleator of apatite formation, and the activated nucleator then forms small apatite crystals near the collagen fibril. Most small apatite crystals (*green*) form complexes with serum fetuin (*red*) that inhibit their growth. A few of these crystals are free at any instant, however, and these can diffuse through pores in the collagen fibril (represented by *gaps* in the *vertical black line*). Because fetuin is too large to pass through the matrix pores, the crystals inside the fibril are free of fetuin and grow rapidly. (Note that the actual ratio of fetuin to crystal is greater than shown and that other inhibitors may substitute for fetuin.)

tity of the serum nucleator that generates these crystals is presently unknown, we have previously demonstrated that it consists of one or more proteins that are 50–150 kDa in size (3, 4). The nucleator is therefore too large to penetrate into the internal aqueous volume of the fibril where mineral deposition occurs. The initial crystals formed by the nucleator can be smaller than a 6-kDa protein, however, and are therefore able to reach rapid diffusion equilibrium with all of the water within collagen fibrils.

3) A source of tissue nonspecific alkaline phosphatase to activate the serum nucleator is required. The results of this study demonstrate for the first time that the nucleator circulates in an inactive form and is activated by tissue-nonspecific alkaline phosphatase. To our knowledge, this is the first direct demonstration that TNAP is absolutely required for the formation of apatite crystals in any system in which phosphate levels are not detectably increased by its phosphomonoesterase activity.

Many previous observations support the hypothesis that tissue-nonspecific alkaline phosphatase plays an important role in normal biomineralization. These include the observation that TNAP is abundantly expressed at sites of bone mineralization (18, 19), where it is held in the outer leaflet of osteoblast and matrix vesicle membranes by a covalently attached glycosylphosphatidylinositol anchor (32), and the observation that genetic deficiency in tissue-nonspecific alkaline phosphatase in mice and humans (called hypophosphatasia) is primarily associated with defects in bone mineralization (14, 16, 17). Despite numerous studies over the past 85 years, however, the molecular basis for the action of TNAP in biomineralization has remained obscure.

Based on our present observations, we propose that the role of TNAP in normal biomineralization is to locally activate the serum nucleator of apatite crystal formation. If this hypothesis is correct, the results of our study provide an assay that can be used to isolate and identify the TNAP substrate that catalyzes the formation of apatite crystals.

4) A large protein (such as fetuin) that selectively inhibits the growth of those crystals remaining in solution outside the matrix is required. This concept is the key to understanding why crystals within the matrix are able to grow, whereas crystals that remain in solution outside of the matrix are not. Fetuin is the major inhibitor of apatite crystal growth in serum (13), and it forms complexes with the small, apatite-like crystals formed at the earliest step in crystal formation, crystals too small to be sedimented by centrifugation, and strongly inhibits their growth (12). Only those crystals that escape fetuin by diffusing into a collagen matrix are free to grow, and by growing they eventually they become too large to diffuse out of the matrix. The selectivity of this calcification mechanism is startling; essentially all of the bulk mineral formed during the incubation in serum lies within the collagen matrix if fetuin is present, or it forms a mineral precipitate if fetuin is absent (6).

Nature of the Serum Nucleator—The mechanism of serum-induced collagen calcification (Fig. 8) contains one major unknown, the identity of the serum nucleator. This is an important topic for future research, and it is useful to summarize what we currently know about the nucleator. In earlier studies, we demonstrated that serum calcification activity consists of molecules that are 50–150 kDa in size (3). The serum nucleator is an essential element of this activity, and so must have this size. We have recently shown that the serum nucleator initiates the formation of numerous apatite-like crystals in serum that is deficient in the apatite growth inhibitor fetuin, crystals that form whether or not a collagen matrix is present (6). In this study, we show that the rate of crystal formation in fetuin-deficient serum is accelerated by the addition of purified TNAP and prevented by the addition of TNAP inhibitors.

Our working hypothesis is that the serum nucleator circulates as an inactive phosphoprotein, and that TNAP activates the nucleator by catalyzing its dephosphorylation.[5] Major objectives of future research will be as follows: to identify the nucleator, to show how it is activated by TNAP, and to determine the mechanism by which the activated nucleator is able to rapidly produce apatite-like crystals at the concentrations of calcium and phosphate that prevail in mammalian serum.

Experimental Evidence That Local Activation of the Serum Nucleator by TNAP Causes Calcification of Type 1 Collagen—The hypothesis that TNAP might initiate collagen calcification at sites of bone formation by locally activating the serum nucleator of apatite crystal formation is supported by the results of

[5] Serum levels of TNAP efficiently dephosphorylate phosphoproteins at alkaline pH (40) as well as at neutral pH (P. A. Price, D. Toroian, and W. S. Chan, personal observations).

two previous investigations. 1) The covalent attachment of TNAP to purified type 1 collagen causes collagen to calcify when implanted at a subcutaneous implant site in a rat (33). This observation shows that blood supplies all but one of the components required for collagen calcification at the subcutaneous implant site, and that this additional component is TNAP. 2) The expression of TNAP in the dermal cells of transgenic mice causes the dramatic and extensive calcification of dermal type 1 collagen (34). This observation shows that blood supplies all but one of the components required for calcification of collagen in the dermal layer of the skin, and that this additional component is TNAP. The results of both studies are consistent with the hypothesis that the mineralization of collagen requires two serum components, fetuin and the inactive form of the nucleator, and two bone components, a layer of type 1 collagen in bone (osteoid) and a high local activity of tissue nonspecific alkaline phosphatase at the mineralizing front.

Is the Molecular Mechanism of Serum-induced Collagen Calcification Relevant to Understanding Normal Bone Mineralization?—Mineralization of endocortical bone tissues in the adult occurs within a vascular compartment called the bone multicellular unit. Blood supplies the bone multicellular unit with the osteoblasts and osteoclasts that form and remove bone as well as the ionic calcium and phosphate that become the mineral phase of bone. Blood also supplies the serum protein fetuin, which accumulates selectively in bone to become a major constituent of the extracellular bone matrix. We propose that blood provides at least one additional component to the mineralization site, the inactive precursor of the nucleator of apatite crystal formation. The major functions of osteoblasts in bone mineralization are to lay down type 1 collagen and to express abundant amounts of TNAP on the osteoblast cell surface to locally activate the serum nucleator to form crystals adjacent to collagen. The fetuin in blood then ensures that only those crystals that diffuse into the collagen fibril are able to grow.

To our knowledge, this is the first integrated hypothesis for the molecular mechanism of bone mineralization that incorporates the following features: 1) a well documented, essential role for TNAP; 2) a demonstrated mechanism for the generation of massive numbers of apatite crystals near collagen (thereby ensuring that each fibril in osteoid will be penetrated by several crystals). 3) a demonstrated mechanism to ensure that crystals grow only within the collagen fibril (Fig. 7).

Although we would argue that the molecular mechanism of serum-induced collagen calcification may be relevant to an understanding of bone mineralization, it is unlikely that serum-induced collagen calcification can be the only mechanism by which bone is mineralized. For example, although genetic defects in tissue-nonspecific alkaline phosphatase are associated with mineralization defects in mice and humans, a substantial amount of collagen still mineralizes in the affected individuals. There must consequently be other methods to initiate mineral formation in bone, and relevance of the serum-induced collagen calcification mechanism may be largely confined to understanding the TNAP-dependent component of the bone mineralization. In addition, whereas fetuin is clearly required for the serum-induced calcification of collagen, there is as yet no evidence that mice that lack fetuin have a defect in bone mineralization. There are certainly other macromolecular inhibitors of apatite crystal growth that are found in bone, some of which may play a similar role to serum fetuin in confining crystal growth to the interior of the collagen fibril. There may also be species differences in the relative importance of different inhibitors, because there is recent evidence that higher fetuin-A levels are independently associated with higher bone mineral density in older women (35), which is the result predicted by the shotgun mineralization mechanism. Finally, to fully understand the possible role of serum-induced collagen calcification in normal bone mineralization, future studies will be needed to compare the mechanical, chemical, and physical properties of native bone to the corresponding properties of demineralized bone that has been re-calcified in serum.

Is the Molecular Mechanism of Serum-induced Calcification Relevant to Understanding Heterotopic Mineralization?—Abnormal calcification of soft tissues is a hallmark of human diseases such as atherosclerosis, diabetes, and uremia. We speculate that the molecular mechanism of serum-induced mineralization could play an important role in the initiation and growth of such abnormal calcifications. This hypothesis is supported by the fact that these calcifications typically occur in close proximity to blood, and they often occur within collagen or elastin fibers. This hypothesis is also supported by the association between high local alkaline phosphatase activity and increased mineral deposition within atherosclerotic plaques (36–38) and at other ectopic calcification sites.

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