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Inhibition of Apatite Formation by Phosphorylated Metabolites and Macromolecules

J. D. Termine and K. M. Conn

Laboratory of Biological Structure, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland

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Apatite formation from synthetic extracellular fluids is rate-limited both at the initial amorphous precursor deposition step and at the amorphous-crystalline transformation reaction. Nucleotide diphosphates and triphosphates and low molecular weight metabolites containing two attached ester phosphate groups all inhibited amorphous-crystalline conversion at concentrations of 10^{-5} to 10^{-6} M. Both native and synthetic polynucleotides as well as the phosphoproteins from rat dentin or egg yolk also inhibited crystal formation from amorphous calcium phosphate. In all cases, substantial amounts of inhibitor molecules were incorporated into the stabilized amorphous precipitates. Treatment of isolated, inhibitor-stabilized amorphous precipitates with hydrolytic enzymes such as alkaline phosphatase or papain reversed the inhibitory effect and permitted crystallization to proceed normally.

Key words: Apatite — Phosphometabolite — Phosphoprotein — Phosphatase.

Introduction

Throughout the development and growth of skeletal tissues, apatite formation is always under rigid biological control with respect to both its kinetic and its spatial aspects. In developing rat dentin, for example, the mineralization front advances about 16 μ per day [9]. Thus, over the entire predentin-dentin calcifying surface, mineral is accumulated at an average rate of more than 100 Å per minute at each accretion site. Furthermore, this process does not occur as random precipitation, but in a rather definitive, three-dimensional fashion so that the resultant crystallites have a concise spatial relationship to their underlying organic matrix.

Obviously, then, under physiologic conditions biological apatites are only formed at a rate, at a place and to an extent consistent with a programmed format controlling the tissue's ultimate composition and structure. The purpose of this study was to investigate exogenously regulated apatite formation from synthetic extracellular fluids in the hope of elucidating possible metabolic control mechanisms that may act on this process in skeletal tissues. A preliminary report of these findings was presented earlier [10].

Materials and Methods

The calcium phosphate formation media used in this study were buffered synthetic extracellular fluids described in detail elsewhere [12]. As before, solution preparation and maintenance procedures were employed that avoided extraneous nucleation effects. All reactions were conducted in 95% O_2–5% CO_2 atmospheres at 37° C and pH 7.40. Under these conditions,

For reprints: Dr. John D. Termine, National Institute of Dental Research, Bldg. 30, Rm. 216, National Institutes of Health, Bethesda, Maryland 20014, USA

apatite crystal formation proceeds from an amorphous calcium phosphate (ACP) precursor at ionic Ca^{+2} and HPO_4^{2-} concentrations close to those found in physiological fluids [12]. In this study, initial Ca^{+2} and HPO_4^{2-} concentrations (4.4 and 3.0 mM, respectively) were used which afforded precipitation within 10–15 min after mixing as a matter of convenience. However, all results were checked at random at still lower supersaturations closer to the physiologic level (e.g., Ca^{+2} and HPO_4^{2-} at 1.6 and 1.1 mM, respectively) to confirm their applicability in tissue systems.

Precipitation was followed by UV turbidimetry and electron microscopy as described previously [12]. The total amount of precipitate formed at any given time was determined from calcium and phosphate analyses [11] of total solution and lyophilized precipitate samples. Crystal formation was assayed by X-ray diffraction and infrared spectrophotometry on Millipore-filtered, freeze-dried aliquots [11, 12]. All additives tested for inhibitory activity were dissolved in phosphate solution at twice their final concentrations prior to mixing at time zero. The mineral formation kinetics referred to in this paper are described elsewhere [11, 12].

In a previous study on pyrophosphate stabilization of ACP, inhibition was reversed upon addition of phosphatase enzyme to the reaction medium [4]. In this investigation, inhibitor-stabilized amorphous precipitates, isolated by Millipore filtration and lyophilization, were resuspended in solutions containing enzymes for which the incorporated or adsorbed inhibitor molecules (see below) were potential substrates. Crystal formation was then followed in the usual fashion. Alkaline phosphatase activity (defined here as reversal of inhibition towards amorphous-crystalline conversion) was assayed from a solution consisting of 0.05 M Tris buffer, 0.10 M NaCl and 0.25 mM $MgCl_2$ at pH 8.50 and 37° C containing 25 μg/ml (10 U) of calf intestine alkaline phosphatase. ACP precipitates were suspended in this system at 250 μg/ml (calcium phosphate). Papain and ribonuclease activity were assayed from a solution consisting of the original precipitation medium minus both calcium and phosphate at pH 7.40 and 37° C in a 95% O_2–5% CO_2 atmosphere and containing either 300 μg/ml (100 U) of papain or 100 μg/ml (500 U) of ribonuclease. ACP precipitates were suspended in this system at 500 μg/ml (of calcium phosphate). Stabilized ACP test precipitates were formed as described above at 5×10^{-4} M concentrations of low molecular weight inhibitors and at either 0.5 mg/ml (phosphoprotein) or 0.1 mg/ml (polynucleotide) levels of high molecular weight inhibitors. The test precipitates were harvested 2 h after formation as described above and then suspended as dry powders into the enzyme medium.

Low molecular weight metabolites were obtained as Na salts from either the Sigma Co. or Calbiochem. Polyadenylic (5′) and soluble ribonucleic acids (RNA) were obtained from Sigma Chemical Co., St. Louis, Mo., phosvitin from Calbiochem, La Jolla, Calif., and Na poly-L-glutamate from Schwarz-Mann, Orangeburg, N.Y. Rat incisor dentin phosphoprotein was a gift from Dr. William T. Butler of the University of Alabama in Birmingham. Alkaline phosphatase and papain were obtained from Boerhinger-Mannheim Biochemicals, Indianapolis, Ind., while ribonuclease was purchased from Worthington Biochemical Corp., Freehold, N. J. Reagent grade chemicals were used throughout.

Results

Figure 1 demonstrates the relative proportion of mineral ions removed from solution during each of the three major events encompassing apatite crystal formation in this in vitro system. The bar labeled *Amorphous Calcium Phosphate Deposition* represents the percentage of solution ions removed during the initial precipitation of precursor solid. The middle bar describes the fraction of solution ions removed during the period where the initial amorphous deposits become transformed into crystals of apatite-like mineral. This is called the amorphous calcium phosphate-crystalline apatite (ACP-CA) conversion period and occurs 8–10 h after the initial mixing of reagents under the routine reaction conditions described above (Ca^{+2} and HPO_4^{2-} at 4.4 and 3.0 mM, respectively). The third bar in this figure represents a subsequent period of crystal proliferation, maturation and growth during which the largest proportion of solution ions are removed,

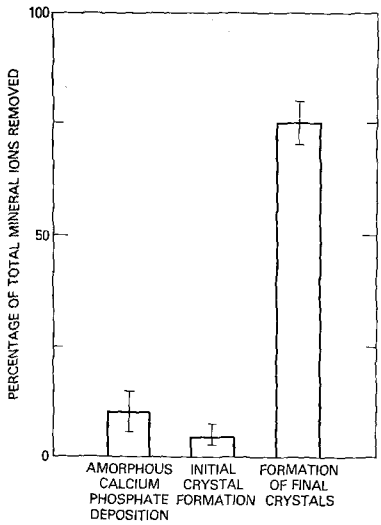

Fig. 1. Percentage of calcium phosphate removed from solution during the three different stages of apatite formation from synthetic extracellular fluids at 37° C, pH 7.40, $(Ca \times P)_0 = 4.1 - 13.5$ mM^2 and under a 95% O_2–5% CO_2 atmosphere. Brackets indicate standard deviations of the mean

Table 1. Effect of added metabolic inhibitors on amorphous/crystalline mineral transformation in synthetic extracellular fluid media[a]

Additive	Conc. (1×10^{-5} M)	Time for ACP-CA conversion (h)
None	—	8–10
2,3 Diphospho-glyceric acid	50	>48
	5	16–20
Fructose 1,6-diphosphate	50	>48
	5	16–18
Glucose 1,6-diphosphate	50	>48
	5	16–20
Adenosine tri-phosphate (ATP)	5	36–48
Adenosine di-phosphate (ADP)	50	>48
	5	14–18
Cytidine di-phosphate (CDP)	50	>48
	5	12–16
Uridine di-phosphate (UDP)	50	30–40

The following had no effect at similar concentrations: 3 phosphoglyceric acid, fructose 6-phosphate, glucose 6-phosphate, adenosine monophosphate (AMP), cyclic AMP, citrate, lactate, pyruvate, succinate, ascorbate

[a] pH = 7.4, 37° C, $(Ca \times P)_0 = 13.5$ mM^2

as shown. Under the conditions described above, this period occurs within 10–24 h after initial reagent mixing. These data indicate that apatite formation from synthetic extracellular fluids is rate-limited at either the initial mineral deposition

Table 2. Effect of added macromolecular inhibitors on amorphous/crystalline mineral transformation in synthetic extracellular fluid media [a]

Additive	Conc. (mg/ml)	Time for ACP-CA Conversion (h)
None	—	8–10
RNA	0.10	>48
	0.05	16–18
Polyadenylic acid (5′)	0.10	>48
	0.05	16–18
Phosvitin	0.50	>48
	0.20	16–20
Rat incisor dentine phosphoprotein	0.50	>48
	0.20	18–24
Poly-L-glutamic acid	0.50	>48
	0.25	40–48
	0.10	20–28

[a] pH = 7.4, 37° C, $(Ca \times P)_0 = 13.5$ mM^2

step or the step at which the initial amorphous precursor transforms into the first seeds of apatite-like crystallites.

Table 1 shows the effect of a number of low molecular weight metabolites on ACP-CA conversion in synthetic extracellular fluids. At the concentrations tested, none of the carboxylic intermediates nor any of the mono-phosphometabolites tested had any effect on this transformation reaction. On the other hand, substantial inhibitory activity was observed in 10^{-5} M concentrations of all the diphosphometabolites tested and all the nucleotides containing at least one pyrophosphate bond. The presence of two pyrophosphate linkages was more inhibitory than a single link (e.g., ATP vs. ADP) and there was some indication that the nucleotide base moiety may have modulated the degree of overall activity in this regard (ADP vs. CDP vs. UDP).

Table 2 shows the effect of added macromolecules on this system. As expected from the metabolite data above, both native (RNA) and synthetic (polyadenylate) polynucleotides showed substantial inhibitory activity toward ACP-CA conversion in synthetic fluids. Although these materials do not contain pyrophosphate linkages, they do have multiple phosphate links in linear array. Similarly then, the phosphoserine-rich native polypeptide specimens, dentin phosphoprotein, and phosvitin inhibited ACP-CA conversion. That such inhibitory activity is not restricted to multiple phosphate sites is demonstrated by the polyglutamate data of Table 2 which shows that multiple carboxyl sites also inhibit the amorphous-crystalline transformation reaction in synthetic media. A similar activity for this material and phosvitin was reported earlier in a less physiologic apatite formation system [13].

In order to determine whether, in the course of their inhibitory activity, these metabolites and macromolecules became adsorbed onto or incorporated within the initial amorphous mineral deposits, infrared spectra were obtained from the stabilized ACP precipitates formed in their presence (data summarized in Fig. 2).

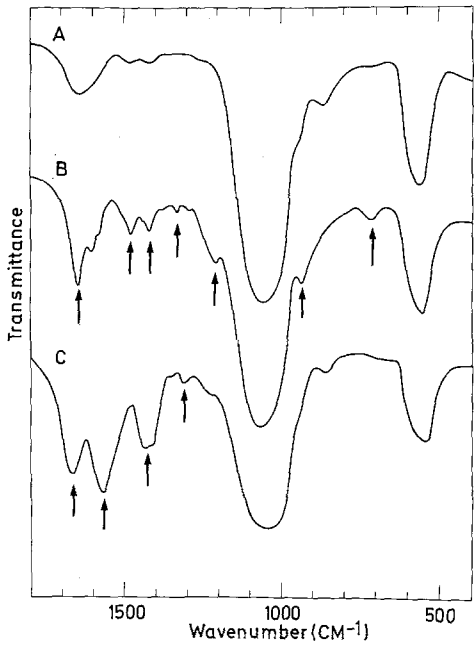

Fig. 2 A—C. Infrared transmission spectra (1800–400 cm^{-1}) of: (*A*) amorphous calcium phosphate (ACP); (*B*) nucleotide (ADP)-stabilized ACP; and (*C*) poly-L-glutamate-stabilized ACP. Arrows indicate inhibitor absorption bands

Table 3. Reversal of inhibition toward amorphous-crystalline conversion by hydrolytic enzymes

Inhibitor used	Enzyme present	Time for ACP-CA conversion (h)	pH[a]
None	Alkaline Phosphatase	24	8.50
2,3 Diphospho-glyceric acid	None	>96	8.50
	Alkaline Phosphatase	24	8.50
Fructose 1,6-diphosphate	None	>96	8.50
	Alkaline Phosphatase	24	8.50
Glucose 1,6-diphosphate	None	>96	8.50
	Alkaline Phosphatase	24	8.50
Adenosine di-phosphate	None	>96	8.50
	Alkaline Phosphatase	48	8.50
Cytidine di-phosphate	None	>96	8.50
	Alkaline Phosphatase	48	8.50
None	Papain, Ribonuclease	4–6	7.40
Phosvitin	None	>96	7.40
	Papain	16	7.40
Polyadenylic acid (5′)	None	>96	7.40
	Ribonuclease	18	7.40

[a] pH of operative solution systems as defined in text. All conversion times given are ± 1–2 h

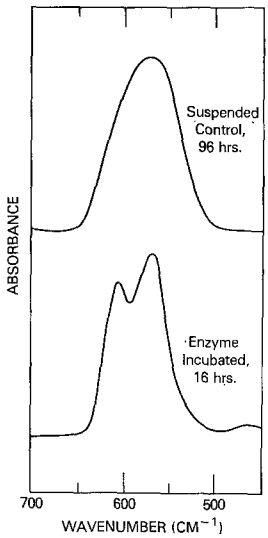

Fig. 3. Linear absorption infrared spectra (700–400 cm^{-1}) of phosvitin-stabilized ACP after 96 h incubation in papain-free media (*top*) and after 16 h incubation at pH 7.40 and 37° C in the presence of papain (*bottom*)

In spectrum B (ADP-stabilized ACP) and spectrum C (polyglutamate-stabilized ACP), arrows point to infrared absorption bands arising from incorporated or adsorbed inhibitor molecules not found in the spectrum of ACP alone (*A*, at the top of Fig. 2). The data depicted were representative of all the inhibitors studied and indicated, in each case, that inhibitor molecules were attached to the stabilized ACP precipitates at levels far in excess of their relative concentrations in free solution. This implies, in turn, a rather high affinity between these materials and ACP.

Table 3 shows the effect of hydrolytic enzymes on crystal formation from presynthesized, inhibitor-stabilized ACP precipitates. Control reactions using pure ACP powders (harvested 2 h after formation) suspended in enzyme solutions gave ACP-CA conversion times consistent with those expected for pure ACP alone at the pH values tested. In the absence of enzyme, the inhibitor-stabilized precipitates remained amorphous for at least 96 h while in the presence of enzyme, crystal formation occurred much earlier. Sample infrared spectra illustrating this behavior are shown in Figure 3 which depicts the PO_4 asymmetric bending mode from lyophilized ACP stabilized with inhibitor (*top*) and from the same material suspended in the presence of enzyme (*bottom*). The absence of splitting in the top spectrum is typical of ACP precipitates, while the double peak in the bottom spectrum signifies the presence of apatite.

Discussion

Apatite formation from extracellular fluid-like solutions is rate-limited both at the initial ACP nucleation step and at the amorphous-crystalline transformation

reaction. Thus, if either of these two steps is inhibited in any way, the overall crystallization process is stopped in its entirety. This investigation reports the inhibition of ACP-CA conversion using metabolites and macromolecules (or their analogues) commonly found in or near cells at mineralizing sites within skeletal tissues.

The generalized nucleotide diphosphate inhibition of crystal formation from amorphous calcium phosphate reported in Table 1 is consistent with the results of a prior study on nucleotide inhibition of seeded apatite crystal growth [8]. However, over the time periods reported in this study, non-enzymatic transphosphorylation of nucleotide high energy phosphate bonds with ACP surfaces could have occurred [2], thus explaining at least part of our nucleotide stabilization data as a resultant mineral-bound pyrophosphate inhibitory effect [4, 5]. Whether this phenomenon occurred in previous experiments on ATP inhibition of apatite formation [1, 7] is uncertain. If inhibitor P—O—P bonds are not present, we found that low molecular weight metabolites containing two ester phosphates (but not monophosphate or carboxylic acid groups) also inhibit ACP-CA conversion. These data, in turn, are consistent with previous reports of crystallization inhibition by phytates [14] and 2,3- diphosphoglycerate [16].

Regardless of the mechanism by which nucleotide or diphosphometabolites inhibit apatite formation from synthetic fluids, they do so at concentrations which approach the physiological. At supersaturations involving Ca^{+2} and HPO_4^{2-} concentrations approximately 2–3 times those of serum or extracellular fluids, substantial inhibition of crystal formation from ACP occurs at 10^{-5} M levels of added low molecular weight inhibitor. Still further, in our spot-check experiments using Ca^{+2} and HPO_4^{2-} levels of 1.6 and 1.1 mM, respectively, inhibition occurred at 10^{-6} M concentrations of added active metabolite or nucleotide. It should also be remembered that these experiments were of the single dose variety. In view of the infrared spectroscopy data above showing a high affinity between ACP and these inhibitor molecules, even greater quantities of inhibitors might well become attached to ACP precipitates formed under physiologic steady-state or "dynamic equilibrium" conditions, thus enhancing their overall inhibitory effects.

The high affinity of ACP for polynucleotides was indicated above from their strong inhibitory effects and by subsequent infrared spectroscopy of polynucleotide-stabilized ACP precipitates. This may point up some inherent dangers that could occur if free mineral were present within cells and suggests strongly that if, as postulated, cells are involved in calcium phosphate transport [6], such mineral solids would have to be packaged or encapsulated in such a way as to protect nucleic acid-containing cell constituents, for example, ribosomal networks.

The inhibition of apatite formation by dentin phosphoprotein noted above is of interest in light of the fact that up to 10% of the developing rat dentin organic matrix consists of this material [3] and that it very rapidly becomes transported to the rat dentin calcification front subsequent to its biosynthesis [17]. This noncollagenous protein is rich in phosphorylated and carboxylated amino acids [3,15] and thus, the finding above that phosphoserine-rich phosvitin and poly-L-glutamic acid both inhibit ACP-CA conversion is consistent with the interpretation that dentin phosphoprotein exerts its inhibitory activity through its acidic side chain groups. That such groups are tightly bound to mineral surfaces in ACP-phos-

phoprotein complexes is indicated by the observation that in contrast to reactions with metabolite- or nucleotide-ACP complexes, both phosvitin- and dentin phosphoprotein-stabilized ACP were not affected by even prolonged alkaline phosphatase treatment.

The data obtained in this study on the enzyme-induced release of inhibitory activity toward ACP-CA conversion demonstrate that it is possible for hydrolytic enzymes to act directly upon calcium phosphate complexes containing targeted inhibitor molecules. This points up two possible ways in which cells could control crystal formation at mineralizing sites. First, a controlled delay in the rate of extracellular apatite formation could be achieved by direct synthesis and secretion of appropriate inhibitor molecules. Such may be the case for dentin phosphoprotein whose steady synthesis during dentin formation could control the speed of crystal formation at the mineralization front. The rate of inhibitor synthesis and extrusion could then regulate the rate at which crystals form in the extracellular matrix. Secondly, cells might control crystal formation by secreting (or not secreting) enzymes that depolymerize or dephosphorylate extracellularly contained inhibitor molecules. Interestingly, alkaline phosphatase abolished the inhibitory activity of all the active low molecular weight metabolites and nucleotides studied above. Also, as noted above, these materials were active inhibitors at physiologic fluid levels. Thus, as other reports have postulated, alkaline phosphatase activity in extracellular matrices might be required to maintain steady mineralization. It would do so by overriding the activity of naturally occurring crystallization inhibitors such as inorganic pyrophosphate or the metabolites examined in this study.

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