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Concentration-Dependent Effect of Phosphate Ester on Apatite Induction *In Vitro*

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Abstract: The effect of immobilized phosphate ester on hydroxyapatite formation was studied *in vitro* using metastable solutions that do not precipitate spontaneously. Phosphophoryn, an acidic phosphoprotein secreted by odontoblast in dentin, was used in this study. Increasing the solution saturation with respect to hydroxyapatite nucleation decreased the mineral induction time (the lag time until mineral formation). Progressive dephosphorylation (50% and 70%) of phosphophoryn by acid phosphatase prolonged the mineral induction time. 95% dephosphorylated phosphophoryn no longer induced mineral formation after 48 hours. The mineral induced by immobilized phosphophoryn was identified to be an apatite by micro-area X-ray diffraction. Using these data and classical nucleation theory, the interfacial tension between the immobilized phosphophoryn and apatite crystal formed on the surface of phosphophoryn was calculated. The interfacial tension on phosphophoryn containing 0.65 mmol phosphate per ml beads was relatively low (89.0 ± 2.0 ergs/cm²), similar to that on hydroxyapatite (91.0 ergs/cm²). Moreover, dephosphorylation of phosphophoryn increased the interfacial tension for hydroxyapatite nucleation to 91.6 ± 1.6 ergs/cm² for 50%-dephosphorylated phosphophoryn, and 96.0 ± 2.4 ergs/cm² for 70%-dephosphorylated phosphophoryn. These results indicate that phosphophoryn possesses a significant potential of apatite nucleation and induces apatite nucleation in a phosphate concentration-dependent manner.

Key words: Immobilized phosphophoryn, concentration-dependent effect, phosphate ester, apatite nucleation, interfacial tension

Introduction

In vertebrates, the major hard tissues such as bone, dentin, cementum, phosphoproteins are thought to play a primary role in the initiation and regulation of mineralization¹⁻⁶⁾. Especially, dentin phosphoprotein (phosphophoryn), a product synthesized by the odontoblast, is accumulated at the mineralization front of dentin matrix, and is strongly associated with the mineral phase⁷⁾. Phosphophoryn has high affinity to calcium ion at physiological pH⁸⁾; moreover, it binds approximately 1.5 additional calcium ions in the presence of phosphate ion⁹⁾. Earlier study has suggested

that phosphophoryn acts as a nucleator or modulator of hydroxyapatite crystal formation consistent with these characteristics²⁾.

Recently, we reported that phosphophoryn purified from bovine dentin and phosvitin (a model phosphoprotein) purified from egg yolk induced apatite formation when added to a metastable solution, only when they were immobilized to insoluble substrate such as type I collagen or agarose beads *in vitro*¹⁰⁻¹²⁾. When these phosphoproteins were electrostatically bound to the above substrates, they did not induce mineral formation but inhibited spontaneous precipitation of calcium phosphate from extremely highly saturated solutions¹⁰⁾. Thus, these observations suggested that the covalently bound phosphophoryn is a strong nucleator of hydroxyapatite formation, and that the electrostatically bound

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phosphophoryn has roles in inhibiting mineralization and regulating mineral growth.

We are interested in defining the role of the substrate surface in hydroxyapatite nucleation. Heterogeneous nucleation reduces the activation energy required for crystal nucleation in a solution that does not precipitate spontaneously. The interfacial tension for hydroxyapatite nucleation is a function of the activation energy to induce hydroxyapatite formation. The interfacial tension between the substrate and the crystalline phase formed on the substrate is an important factor controlling the nucleation process¹³⁻¹⁵. Thus, it is a good measure of the hydroxyapatite induction potential of a surface^{14,16,17}. When the surface of a solid substrate matches well with the crystalline phase, interfacial tension becomes very small. We previously reported an interfacial tension of 91.5 ergs/cm² for hydroxyapatite nucleation on insoluble dentin collagen containing collagen-bound phosphophoryn¹⁰, and this value was similar to the interfacial tension reported by Christoffersen et al.¹⁸ (91.0 ergs/cm²) for hydroxyapatite nucleation by hydroxyapatite seeding. Therefore, we postulated that highly phosphorylated surface have matching interfacial tension for hydroxyapatite nucleation¹⁰.

In previous studies, we also found that the time to apatite formation induced by immobilized phosphoprotein was prolonged with the progressive dephosphorylation of phosphoprotein^{10,11}. However, the role that phosphate ester in immobilized phosphoprotein plays in apatite nucleation remains unclear.

Our aim in this study was to determine the effect of concentration of phosphate ester on the apatite nucleation potential of immobilized phosphoprotein, by means of calculation of interfacial tension.

Materials and Methods

Preparation of Phosphophoryn-Agarose Beads

Phosphophoryn was prepared from molar teeth extracted from 8 to 9-month-old calf jaws following the methods described by Butler¹⁹. The preparation was carried out at 4°C in solutions to which protease inhibitors (100 mM 6-aminohexanoic acid, 5 mM benzamidinium-HCl, and 1 mM phenylmethylsulfonyl fluoride) were added. Phosphophoryn was purified through an anion exchange chromatography step. Each preparation was sampled for amino acid analysis and phosphate analysis using malachite green²⁰ after alkaline hydrolysis.

Phosphophoryn was immobilized onto agarose beads (Sephacrose 4B, Pharmacia Fine Chemicals, Uppsala, Sweden) using divinyl sulfone (Sigma Chemical Co., St. Louis, MO). The amount of phosphate ester immobilized to agarose beads was determined by a phosphate assay.

Dephosphorylation of Immobilized Phosphophoryn

Phosphophoryn covalently cross-linked to agarose beads was partially dephosphorylated using potato acid phosphatase (Sigma Chemical Co.) in 10 mM sodium acetate buffer containing 50 µM

EDTA, pH 5.8 at 37°C^{21,22}. After obtaining a standard curve on the relation between treatment period and degree of dephosphorylation, the time of acid phosphatase treatment of phosphophoryn cross-linked to agarose beads was determined for the mineralization experiment. The amount of residual organic phosphate ester was measured in the dephosphorylated phosphophoryn.

Hydroxyapatite induction

The compositions of the metastable calcium phosphate solutions used in this study are shown in Table 1. All solutions contained 10 mM HEPES-KOH for buffering at pH 7.40 at 37°C, and the final ionic strength was adjusted to 0.16 with KCl. The degrees of saturation with respect to hydroxyapatite were calculated using the software program WATEQ4F^{2,3}, after modification to include the contributions of the HEPES buffer and sodium azide to the ion strength. The partial pressure of carbon dioxide in all solutions was assumed to be 3×10^{-4} atmosphere. WATEQ4F includes calculations of complex formation, ion concentrations and activity coefficient of carbonic acid, and its dissociation products in the calculations of mineral saturation. The solutions were filtered through a 0.22-µm filter.

Ten microliters of phosphophoryn or dephosphorylated phosphophoryn cross-linked to agarose beads were incubated in 3 ml of the metastable solutions at 37°C in a shaker (ADVANTEC TOYO, TS-200, Tokyo) operated at 125 rpm. Samples were collected at several time points and filtered. The residue on the filter was dissolved in 0.1 N HCl containing 0.25% lanthanum chloride. Then, the samples were analyzed for calcium by atomic absorption spectrometry (Perkin-Elmer, Model 5100, Norwalk, Conn, U.S.A.).

Mineral induction time (the lag time until mineral is formed) was determined from a plot of Ca (µg) vs. incubation time (sec). Using the least-squares method, a line was drawn to fit the data for amounts of calcium deposited on the beads not exceeding that at zero time point. A second line by least-squares was drawn to fit the remaining data. Induction time was taken as the interval from the addition of the mineralization medium to the point at which these lines intersected. Using these data, a slope of the plot of log (induction time) vs. (log saturation)⁻² was identified. The value of this slope was used to calculate the interfacial tension for hydroxyapatite nucleation on phosphophoryn cross-linked to agarose beads.

Interfacial Tension

The interfacial tension (σ), as described by classical nucleation theory²⁴, was calculated as follows:

$$\sigma = 2.303kT[(5\alpha)/2\beta v^2]^{1/3} \quad (1)$$

where σ = interfacial tension

k = Boltzman constant (1.38×10^{-6} ergs/deg.)

T = temperature in degrees Kelvin (310.15°K)

α = slope of log (mineral induction time) versus (log saturation)⁻²

Table 1. Composition of metastable calcium-phosphate solutions

[Ca][PO ₄] (mM ²)	CaCl ₂ (mM ²)	K-PO ₄ (mM ²)	Hepes (mM ²)	KCl (mM ²)	NaN ₃ (mM ²)	Saturation Log (IAP/Ksp)
3.30	3.35	1.41	10	150	3.08	7.74
3.15	2.29	1.37	10	150	3.08	7.65
3.00	2.24	1.34	10	150	3.08	7.59
2.70	2.20	1.34	10	150	3.08	7.53

Figure 1. Mineral induction by intact phosphophoryn immobilized to agarose beads (0.65 μmol phosphate/ ml beads). Using the least-squares method a line was drawn to fit the amounts of calcium bound from the medium was within one standard deviation of that at the zero time point. A second least-squares line was drawn to fit the remaining data. Induction time was taken as the interval between the addition of the mineralization solution and the point at which these lines intersected. Phosphophoryn immobilized to agarose beads (0.65 μmol phosphate/ ml beads) was incubated at 37°C in metastable solution having saturation

Figure 2. Mineral induction by phosphophoryn immobilized to agarose beads at 37°C in metastable solution having saturation with respect to hydroxyapatite of 7.59. Data for immobilized phosphophoryn having phosphate ester of 0.65 μmol / ml beads (intact) (square), 0.33 μmol / ml beads (50%-dephosphorylated) (circle), 0.20 μmol / ml beads (70%-dephosphorylated) (triangle) and 0.03 μmol / ml beads (95%-dephosphorylated) (x) are shown.

β = a dimensionless geometric factor ($16\pi/3$ for spherical)

v = molecular volume of hydroxyapatite ($2.8 \times 10^{-22} \text{ cm}^3$)

Scanning Electron Microscopy

After twenty-four hour incubation in a solution at saturation of 7.59, the mineral formed by phosphophoryn cross-linked to agarose beads was collected by filtration onto 0.2 μm polycarbonate membrane, rinsed with water adjusted to pH 10 with NH_4OH , coated with gold, and observed under a JEOL JSM-T100 scanning electron microscope.

Micro Area X-ray Diffraction

Mineral formed after 24 hour incubation was rinsed with water adjusted to pH 10 with NH_4OH , dried, and analyzed with RIGAKU RINTO-2000 X-ray diffractometer (Cu $K\alpha$, 50 kV, 25 mA).

Results

After covalent cross-linking of phosphophoryn to agarose beads, 0.65 ± 0.03 (mean $\pm$ SD, $n=6$) μmol of phosphate ester per ml

beads was bound to agarose beads. Intact phosphophoryn was dephosphorylated using acid phosphatase, and three degrees (49.7%, 70.4%, and 95.0% dephosphorylation) of dephosphorylated phosphophoryn were obtained. The samples were examined for in vitro mineral induction in 3 ml of metastable solution. No mineral formation was induced by agarose beads alone. Intact phosphophoryn cross-linked to agarose beads induced mineral formation, and the mineral induction time was shortened with increasing degree of saturation (Fig. 1). Fifty percent- and seventy percent-dephosphorylated phosphophoryn that had been immobilized onto agarose beads induced mineral formation similar to intact phosphophoryn (Fig. 2). With progressive dephosphorylation of immobilized phosphophoryn, however, the mineral induction time was prolonged (Fig. 2). Ninety-five percent-dephosphorylated phosphophoryn no longer induced mineral formation.

Examples of the behavior of the crystal nucleated on phosphophoryn cross-linked to agarose beads are shown in Fig. 3. Plate-like crystals formation was induced in all samples. Apatite was the only mineral in phosphophoryn cross-linked to agarose beads detected by micro-area X-ray diffraction (Fig. 4). No

diffraction line corresponding to the 100 plane of octacalcium phosphate was detected.

The mineral induction times for phosphophoryn in metastable solutions at different degrees of saturation with respect to

hydroxyapatite were determined. The plots of $(\log \text{saturation})^{-2}$ versus $\log (\text{induction time})$ is shown in Fig. 5. These slopes were

Figure 3. Scanning electron micrographs of crystals induced by intact (A), 50%-dephosphorylated (B), and 70%-dephosphorylated (C) phosphophoryn immobilized to agarose beads for 24 hours at 37°C in metastable solution having saturation with respect to hydroxyapatite of 7.59. Bar represents 10 μm .

Figure 4. X-ray diffraction patterns of crystals induced by intact (A), 50%-dephosphorylated (B), and 70%-dephosphorylated (C) phosphophoryn immobilized to agarose beads for 24 hours at 37°C in metastable solution having saturation with respect to hydroxyapatite of 7.59, and of hydroxyapatite (#082-04245, Wako Chem. Co., Tokyo) (D). The diffraction maxima by the two major diffraction planes (002 and 211, in order of increasing angle) are distinct.

Figure 5. Plot for the determination of the interfacial tensions for nucleation on intact (square), 50%-dephosphorylated (circle), and 70%-dephosphorylated (triangle) phosphophoryn immobilized to agarose beads. The slopes of the lines were used to calculate the interfacial tensions.

used for calculation of interfacial tension for hydroxyapatite induction on phosphophoryn. The interfacial tension for hydroxyapatite nucleation on immobilized phosphophoryn with 0.65 mmol phosphate ester per ml beads was 89.0 ± 2.0 ergs/cm². After dephosphorylation of the phosphophoryn cross-linked to agarose beads, the interfacial tension was increased to 91.6 ± 1.6 ergs/cm² for hydroxyapatite nucleation on 50%-dephosphorylated phosphophoryn cross-linked to agarose beads (0.33 mmol/ml beads), and 96.0 ± 2.4 ergs/cm² for 70%-dephosphorylated phosphophoryn (0.20 mmol/ml beads).

Discussion

Our observations confirmed that phosphoproteins have high ability to induce apatite formation from metastable solutions that do not precipitate spontaneously, when the phosphoprotein is covalently cross-linked to insoluble substrate; and that the ability is comparable to that of hydroxyapatite seeded crystals as shown by calculating the interfacial tension for hydroxyapatite nucleation. We previously reported that anionic phosphoproteins strongly bound to calcium ions may be potent regulator of crystal growth in solution, and are efficient nucleators when bound to a solid substrate¹⁰⁻¹². This study also shows that immobilization of the phosphoprotein to a certain substrate is necessary to induce mineral nucleation. Mineral ion clusters formed in the metastable solution or on the surface of the nucleator would bind to the surface and express many different short-range orders. One of these orders might match a configuration available on the immobilized phosphoprotein, which stabilizes the cluster to promote the formation and growth of a crystalline order into an insoluble network. On the other hand, Marsh⁹ observed that phosphoprotein alone did not promote the conversion of the sequestered calcium-phosphate clusters into crystalline form, and concluded that this failure might be the result of self-association of free phosphoprotein molecules in solutions containing calcium and phosphate. Thus, phosphoproteins seem to have dual roles in the mineralization of dentin and bone.

Studies using *in vitro* gel diffusion systems reported that low concentrations of phosphoprotein promoted hydroxyapatite formation, whereas higher concentrations inhibited hydroxyapatite growth^{25,26}. However, since spontaneous precipitation occurred in these systems, the analyses might have been on modification of mineral growth rather than mineral nucleation. In our *in vitro* system using metastable solutions in which spontaneous precipitation does not occur, induction of mineral nucleation by the immobilized phosphophoryn can be observed clearly.

In a previous study, we found that the time of apatite nucleation induced by phosphoprotein was prolonged with progressive dephosphorylation of phosphoprotein. In the present study, we examined the effect of the degree of dephosphorylation on the mineral induction potential of phosphophoryn by calculating the interfacial tension. Comparison of the interfacial tension between

intact and dephosphorylated phosphophoryn showed that the interfacial tension increased with progressive dephosphorylation of phosphophoryn within a close range of 89-96 ergs/cm². This close range of interfacial tension shows that phosphophoryn is an effective nucleator of apatite.

The present results indicate that immobilized phosphophoryn possesses a very high potential of apatite nucleation, that the apatite nucleation potential of phosphophoryn is dependent on the degree of phosphorylation, and that a minimal amount of phosphate ester is required for mineral nucleation. Finally we conclude that the phosphate esters associated with immobilized phosphoprotein play an important role in the induction of apatite nucleation on the phosphoprotein surface.

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