〔ORIGINAL〕

In vitro mineral induction by immobilized phosphoprotein
—Effect of phosphate group on interfacial tension for mineral induction—

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Abstract

Phosphoproteins are thought to play a primary role in the deposition of mineral on the collagen of dentin. We previously reported that immobilized phosphoproteins reduced activation energy for mineral induction in metastable mineralizing solutions, and that phosphate ester was essential for mineral induction. Interfacial tension is a good measure to evalute the mineral induction potential because it is a function of the activation energy.

The purpose of this study was to determine the effect of phosphate ester on interfacial tension for mineral nucleation by immobilized phosphoprotein. Phosvitin from egg yolk was used as a model phosphoprotein in this study. Phosvitin was cross-linked to agarose beads with divinyl sulfone. A portion of the cross-linked phosvitin was partially dephosphorylated with potato acid phosphatase. Then, samples were incubated at 37°C in metastable solutions that do not spontaneously precipitate, and the mineral induction time was determined in the samples. The mineral formed was confirmed by X-ray diffraction to be hydroxyapatite. Using classical nucleation theory, the interfacial tension for hydroxyapatite nucleation by intact phosvitin was determined to be 91.3ergs/cm^2, it was 93.2 ergs/cm^2 for 50%-dephosphorylated phosvitin, and 98.1 ergs/cm^2 for 70%-dephosphorylated phosvitin. Mineral formation was not induced by phosvitin that had been 94%-dephosphorylated. These results indicate that the potential of hydroxyapatite nucleation of phosvitin is high as long as it has a minimum number of phosphate esters for hydroxyapatite nucleation, and that the interfacial tension for hydroxyapatite nucleation by immobilized phosphoprotein is dependent on the degree of phosphorylation.

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Introduction

Acidic macromolecles including phosphoglycoproteins, sulfated carbohydrates, acidic proteins, and acidic phospholipids have been associated with biomineralization *in vivo*[1-12]. An understanding of how acidic macromolecules control induction and growth of mineral has potential applications in medicine and biomaterials science. We have been focusing on phosphoproteins which have been considered to have a primary role in nucleation of mineral in dentin[4-9]. When phosphoproteins are free in solution they inhibit mineral nucleation and crystal growth on apatite from spontaneously precipitating solutions. However, when they are immobilized on insoluble substrates such as agarose beads and collagen fibrils, they induce mineral formation from metastable solutions that do not spontaneously precipitate[4-8]. It was found that mineral induction time increased with progressive dephosphorylation of immobilized phosphoprotein, and it was concluded that phosphate esters in phosphoprotein are required for mineral nucleation[4].

We are interested in defining the role of the surface in mineral induction. The interfacial tension for mineral nucleation is a good measure of the mineral induction potential of a surface[13]. We previously reported that interfacial tension for hydroxyapatite nucleation on phosphophoryn was 90.1 ergs/cm^2[-5], similar to the interfacial tension (91.0 ergs/cm^2) for hydroxyapatite nucleation on hydroxyapatite determined by Christoffersen *et al.*[13]. This led to the assumption that highly phosphorylated surfaces have similar interfacial tension for hydroxyapatite nucleation.

The objective of this study was to determine whether interfacial tension for hydroxyapatite nucleation on immobilized phosphoprotein depends on phosphate ester.

Materials and methods

1. Immobilization of Phosphoprotein to Agarose Beads

Phosvitin (Sigma Chemical Co., St. Louis, MO) was used as a model phosphoprotein in this study. It has a 35 kDa apparent molecular weight, and contains 57% serine + phosphoserine, 9.1% -isoleusine, 6.8% -lysine, and the phosphate content is 24.6% (w/w). Phosvitin was immobilized to agarose beads (Sepharose 4B, Pharmacia Fine Chemicals, Uppsala, Sweden) with divinyl sulfone (Sigma Chemical Co., St. Louis, MO)[15]. The amount of phosvitin immobilized to agarose beads was determined by phosphate assay with Malachite Green[16].

2. Dephosphorylation of Immobilized Phosphoprotein

Phosvitin covalently crosslinked to agarose beads was partially dephosphorylated with potato acid phosphatase (Sigma Chemical Co.) in 10 mM Sodium acetate buffer containing 50 μM EDTA, pH 5.8 at 37°C[17]. The amount of remaining organic phosphate ester was

measured in dephosphorylated phosvitin.

3. Mineral Induction Experiment

The compositions of the metastable calcium phosphate solutions that were used in this study are shown in Table 1. The degrees of solution saturations [log (ion activity product) / log (activity product at saturation)] with respect to hydroxyapatite were calculated using computer program WATEQ4F[18]. Mineralizing solution saturations of 7.41, 7.53, 7.59 and 7.74 were used. In all cases, the mineralizing solution had a molar Ca/P of 1.67, contained 10 mM Hepes -KOH for buffering at pH 7.40 at 37°C, and 0.02% sodium azide to prevent bacterial growth, and had a final ionic strength adjusted to 0.16 with KCl. The solutions were filtered through a 0.22 μm filter. None of these solutions spontaneously precipitated, even when held at 37°C for seven days.

Table1 Composition of metastable calcium phosphate solution

[Ca][P] (mM^2)	Ca (mM)	PO_4 (mM)	KCl (mM)	Hepes (mM)	Saturation
3.30	2.35	1.41	150	10	7.782
3.15	2.29	1.37	150	10	7.650
3.00	2.24	1.34	150	10	7.585
2.70	2.12	1.27	150	10	7.411

Ten microliters of each sample 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 taken at several time points and filtered. The filtrate was dissolved in 0.1N HCl containing 0.25% lanthanun chloride. Then, samples were analyzed for calcium by atomic absorption spectrometry (Perkin-Elmer, Model 5100, Norwalk, Conn, U.S.A.).

The induction time was determined from a plot of Ca (μg) vs. incubation time (sec). Using this information, 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.

4. Interfacial Tension

Mineral nucleators reduce the free energy required for nucleation, then facilitating crystal nucleation from solutions that do not spontaneously precipitate. The interfacial tension between the substrate and the crystalline phase formed is a good measure of the nucleation potential of the substrate[19]. The interfacial tension (σ), as described by classical nucleation theory[20], was calculated as follows:

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

σ = interfacial tension

k = Boltzmann's constant (1.38×10^{-6} ergs/deg)

T = temperature in degrees Kelvin (310.15°K)

α = slope of log (induction time) vs. (log saturation)$^{-2}$

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

v = molecular volume of hydroxyapatite (2.8×10^{-22})

5. Scanning Electron Microscopy

Mineral formed was collected by filtration onto a 0.2μm polycarbonate membrane, rinced with water adjusted to pH 10 with NH_4OH, coated with gold, and observed in a JEOL JSM -T100.

6. Micro Area X-ray Diffraction

Mineral formed was rinsed with water adjusted to pH 10 with NH_4OH, dried, and identified using RIGAKU RINTO-2000 (Cu Kα, 50kV, 25mA).

Results

The phosphate determination showed that the phosvitin covalently crosslinked to agarose beads was 12.2μg /μl beads.

Intact and three degrees (49.5%, 71.1% and 94.0%) of dephosphorylated phosvitin were obtained. The intact phosvitin, 50%- and 70%-dephosphorylated phosvitin induced mineral

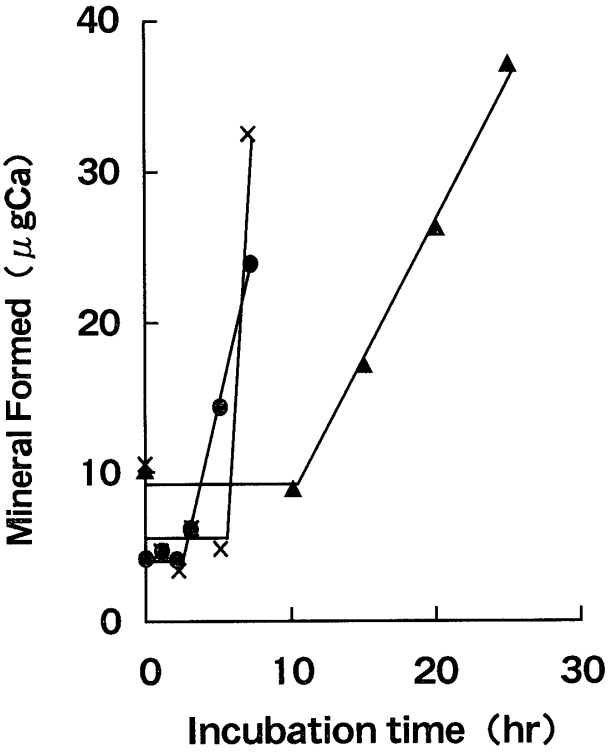

Fig. 1 Mineral induction by intact PV-Agarose beads

Determination of mineral induction times by intact phosvitin cross-linked to agarose beads in metastable solution having the saturation of 7.74 (●), 7.59 (×), or 7.53 (▲).

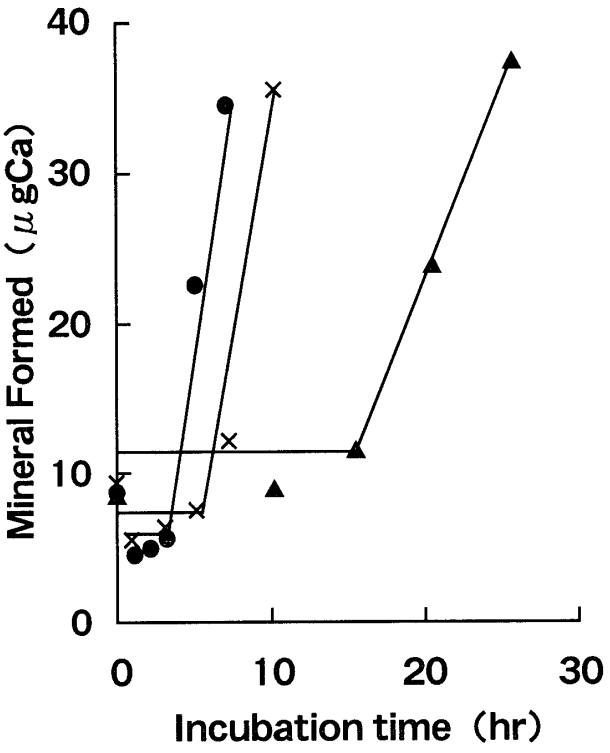

Fig. 2 Mineral induction by 50%-Dep PV-Agarose beads

Determination of mineral induction times by 50%-dephosphorylated Phosvitin cross-linked to agarose beads in metastable solution having the saturation of 7.74 (●), 7.59 (×), or 7.53 (▲).

formation in the mineralizing solution (Figure 1, 2, and 3), while the 94%-dephosphorylated phosvitin did not induce mineral formation. Increasing the solution saturation decreased mineral induction time. Also, mineral induction time increased with progressive dephosphorylation of phosphoprotein.

The interfacial tension for each of these preparations was calculated from the slope of a plot of log (induction time) vs. (log saturation)$^{-2}$ (Fig. 4). Interfacial tension for hydroxyapatite nucleation on intact phosvitin was 91.3 ergs/cm^2, it was 93.2 ergs/cm^2 for 50%-dephosphorylated phosvitin, and 98.1 ergs/cm^2 for 70%-dephosphorylated phosvitin (Table 2).

Electron microscopic observations showed that large crystals were formed on the surface of agarose beads in intact, 50%-, and 70%-dephosphorylated phosvitin, and that crystals were not formed with the 94%-dephosphorylated phosvitin (Fig. 5).

Micro area X-ray diffraction showed that the crystals formed were all hydroxyapatite (Fig. 6). The peaks were broadened, suggesting carbonate-containing or calcium-deficient hydroxyapatite was formed.

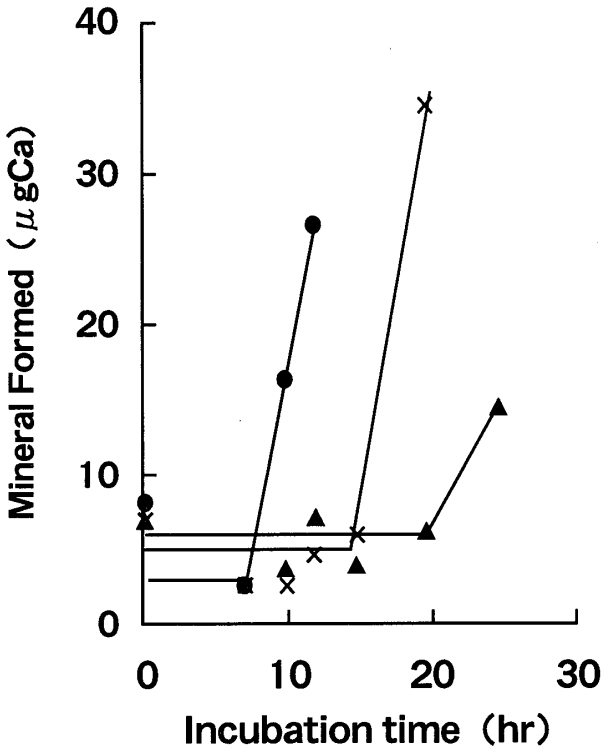

Fig. 3 Mineral induction by 70%-Dep PV-Agarose beads
Determination of mineral induction times by 70%-dephosphorylated Phosvitin cross-linked to agarose beads in metastable solution having the saturation of 7.74 (●), 7.66 (×), or 7.59 (▲).

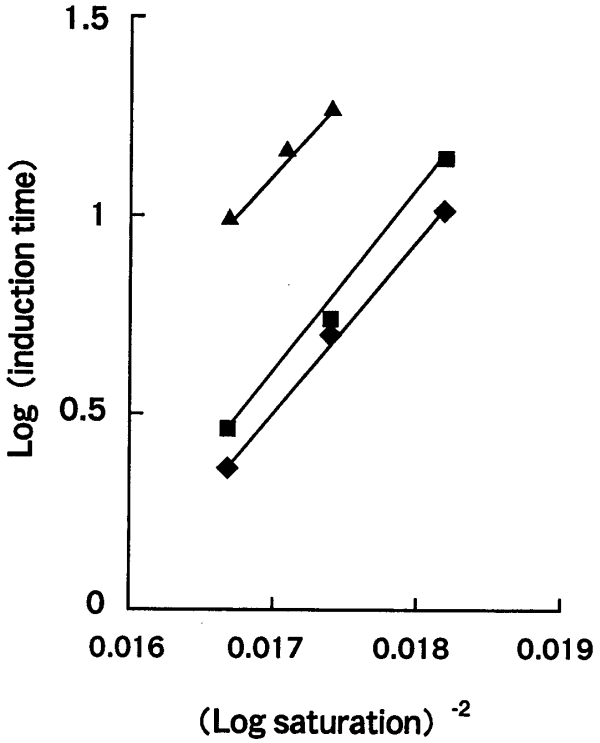

Fig. 4 Plot for determination of the interfacial tension
Plot for determination for interfacial tension. The slopes of the lines for intact phosvitin (◆), 50%-(■), and 70%-dephosphorylated phosvitin (▲) were used to calculate the interfacial tension.

Table2 Interfacial tension for hydroxyapatite nucleation on phosvitin

	Interfacial Tension (ergs/cm²)
intact-PV	91.3
50%Dep-PV	93.2
70%Dep-PV	98.1

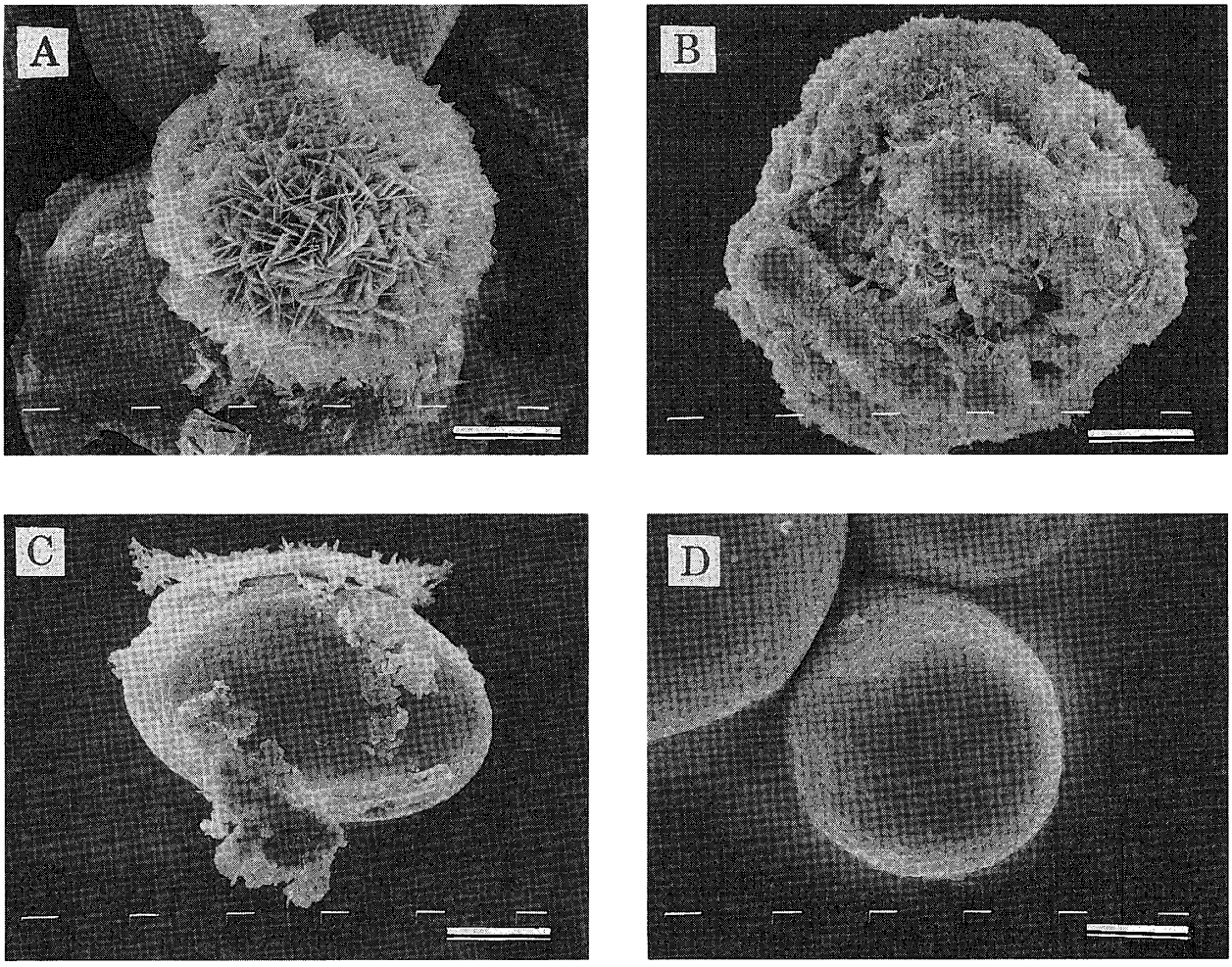

Fig. 5 SEM photographs of mineral formed on phosvitin cross-linked to agarose beads one day after incubation in the solution with saturation 7.59. The crystalline clusters were formed on the surface of agarose beads in (a) intact phosvitin, (b) 50%-dephosdphorylated phosvitin, and (c) 70%-dephosphorylated phosvitin, (d) 94%-dephosphorylated phosvitin cross-linked to agarose beads did not induce mineral formation. (Bar=10μm)

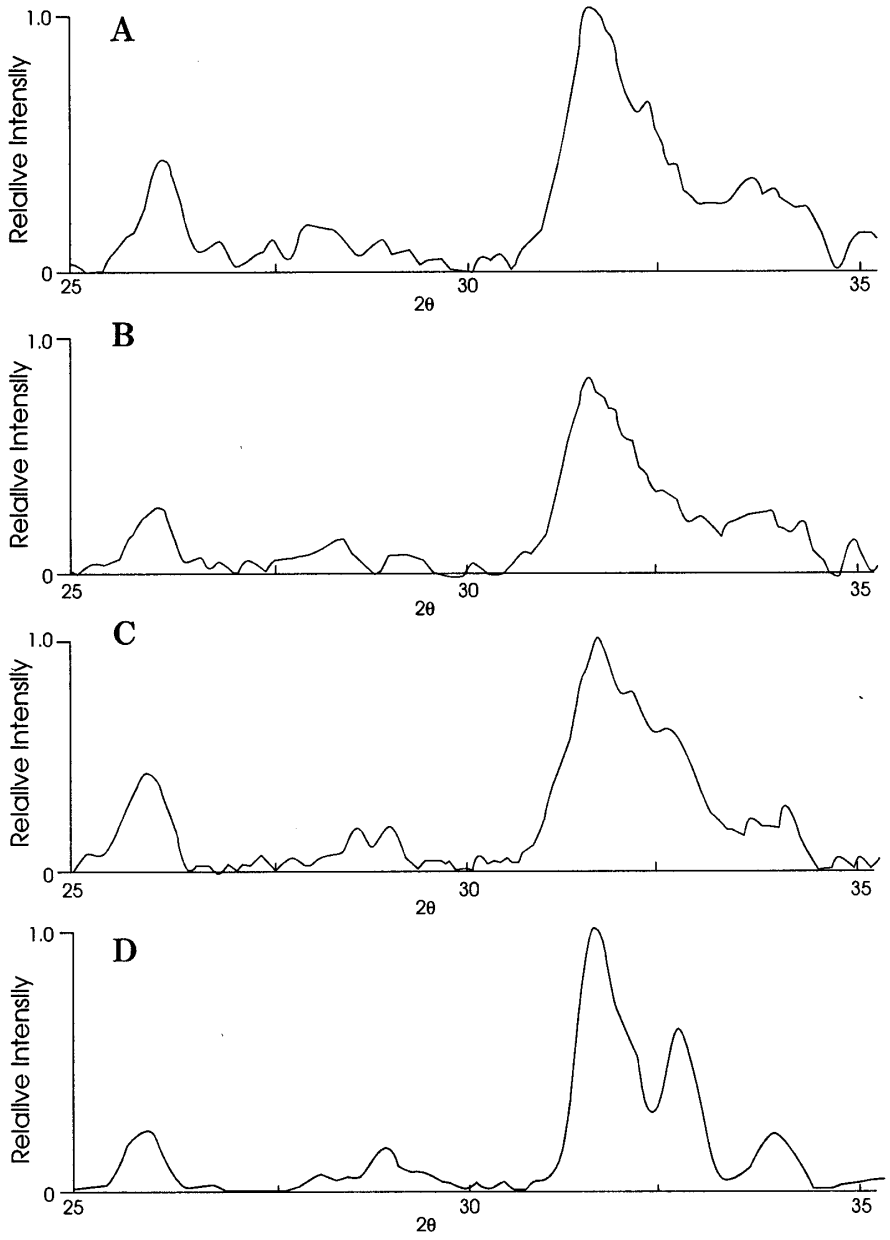

Fig. 6 Micro-area X-ray diffraction pattern of mineral formed by (A) intact phosvitin, (B) 50%-dephosphorylated phosvitin, and (C) 70%-dephosphorylated phosvitin cross-linked to agarose beads, and (D) hydroxyapatite.

Discussion

Previously, we reported that immobilized phosphoproteins induced mineral formation while free phosphoproteins did not induce from metastable calcium phosphate solutions[4-9]. These findings suggested that immobilized phosphoproteins bind most strongly and in a stereospecific way to calcium phosphate complexes that present a complementary constellation of surface charges. On the other hand, free phosphoproteins self-associate, and this may make the overall free energy of binding to their complementary surfaces decrease.

In the study here, we confirmed that a minimum amount of phosphate ester is required for mineral nucleation in immobilized phosphoprotein. The mineral formed by immobilized phosvitin was confirmed as a hydroxyapatite by X-ray diffraction pattern.

Heterogeneous nucleation reduces the activation energy required for crystal nucleation in the solution that does not precipitate spontaneously. The interfacial tension between the substrate and the crystalline phase formed is the most important factor controlling the crystal nucleation process. When the surface of the solid substrate match well with the crystalline phase, interfacial tension becomes very small[13].

In the present study, increasing the solution saturation decreased mineral induction time with regard to intact, 50%-, and 70%-dephosphorylated phosvitin. Using the data concerning mineral induction time and the classical nucleation theory[20], the interfacial tension for hydroxyapatite nucleation on intact phosvitin was determined to be 91.3 ergs/cm^2. This value is consistent with that that we reported previously[4-5]. With 50%-dephosphorylated phosvitin, it was 93.2 ergs/cm^2, and it was 98.1 ergs/cm^2 for 70%-dephosphorylated phosvitin. However, hydroxyapatite nucleation was not induced by 94%-dephosphorylated phosvitin. The interfacial tension for hydroxyapatite nucleation on hydroxyapatite has been reported as 91.0 ergs/cm^2, and for fluoroapatite, as 113.0 ergs/cm^2[-14]. Therefore, the interfacial tensions that were obtained in this study were dependent on degree of phosphorylation but still similar to that on hydroxyapatite.

This study indicates that interfacial tension for hydroxyapatite nucleation on phosvitin is dependent on the degree of phosphorylation, and that the potential of hydroxyapatite nucleation of dephosphorylated phosvitin is still high as long as phosphorylation but has a minimum number of phosphate esters for hydroxyapatite nucleation.

Conclusions

1. Mineral induction times increased with progressive dephosphorylation of phosvitin. Mineral formation was not induced by phosvitin that was 94%-dephosphorylated. This shows that a minimum number of phosphate esters are required for mineral induction.
2. The interfacial tension for hydroxyapatite nucleation on intact phosvitin was determined to be 91.3 ergs/cm^2. For 50%-dephosphorylated phosvitin, it was 93.2 ergs/cm^2, and it was 98.1 ergs/cm^2 for 70%-dephosphorylated phosvitin. The interfacial tension for hydroxyapatite nucleation by immobilized phosvitin is dependent on the degree of phosphorylation.
3. The potential of hydroxyapatite nucleation of phosvitin is high as long as phosvitin has a minimum number of phosphate esters for hydroxyapatite nucleation.

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