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Binding of Dentin Noncollagenous Matrix Proteins to Biological Mineral Crystals: An Atomic Force Microscopy Study

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Abstract. Noncollagenous matrix proteins (NCPs) of dental hard tissues (dentin, cementum) are involved, both temporally and spatially, in the mineralization of their collagen matrices. Two of the NCPs thought to initiate mineral nucleation and control crystal growth in dentin, are dentin phosphoproteins (DPP) and dentin sialoprotein (DSP). Control of crystal growth would depend on the binding capacity of these two molecules, which may be related to the charge domains on the crystals and/or the phosphorylation of the protein. Phosphophoryn (a highly phosphorylated DPP) and DSP were isolated, purified, and characterized from the immature root apicies of human teeth. Dephosphorylation of phosphophoryn was carried out using bovine intestinal alkaline phosphatase. Enamel crystals were prepared from the maturation stage of developing rat incisor enamel. Protein-coated crystals were prepared for viewing in an atomic force microscope fluid cell using tapping mode. Desorption of the proteins was achieved using a phosphate buffer and surface roughness measurements were obtained from all specimens. Time-lapsed images of the crystals showed "nanospheres" of protein distributed along the crystals but only the phosphophoryn-coated crystals showed a distinctive banding pattern, which was still visible after the phosphate desorption experiments. The surface roughness measurements were statistically greater ($P < 0.01$) when compared to the control for only the phosphophoryn-coated specimens. It is hypothesized that the phosphophoryn binding may be associated with charge arrays on the crystal surface and its phosphorylation. Also, based on its affinity for the crystalsurfaces, phosphophoryn seems the most likely candidate for controlling dentin crystal growth and morphology.

Key words: Dentin — Protein — AFM — Binding — Crystals

The successful mineralization of the collagen matrix of dental hard tissues (dentin, cementum) during tooth development and repair is exquisitely dependent on the sequencing of a series of genetic and biochemical events. The noncollagenous matrix proteins (NCPs) of these tissues are intimately involved, both temporally and spatially in mineralization, including the processes of mineral nucleation and control of crystal growth [1]. In these later processes, one group of NCPs, dentin phosphoproteins (DPPs), have been the subject of considerable scientific attention because of their apparent ability to stimulate or inhibit mineral formation [2–6].

Dentin sialoprotein (DSP) has been suggested to have a function in dentin mineralization [2], but its precise role is currently unknown. However, DSP mRNA expression and secretion of the protein itself are associated with dentin mineral formation [7]. In addition, the existence of a single gene coding for both DSP and DPP suggests that the functional roles of these two NCPs may also be tightly coupled with respect to dentin mineral nucleation [8].

A highly phosphorylated DPP, phosphophoryn, has been isolated from mammalian dentin, including human tissue [9–12], and is comparatively abundant in dentin (> 50% in the rat, possibly less in humans). Phosphophoryn, by convention, has > 75–80% of its amino acid residues as aspartic acid and (phos) serine residues, and under conditions of physiological pH and ionic strength, phosphophoryn will nucleate apatite crystals [3, 4, 5]. This is perhaps, related to phosphophoryn acting as a template for biomineralization. However, its activity may be dependent on its molecular structure, that is, whether it is folded or unfolded, which in turn may be pH-dependent [13]. It has also been reported that phosphophoryn binds to the gap region of Type 1 collagen fibrils and initiates plate-like apatite crystal formation [14]. Further, phosphophoryn has been suggested to affect crystal growth and so influence crystal morphology by several but probably related mechanisms: by binding to ions or ion clusters; stabilizing the crystal nucleus; binding to the 100 face of growing apatite crystals; and/or binding to multiple growth sites [15–18].

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It has also been suggested that the degree of phosphorylation of phosphophoryn has marked effect on both collagen Type I fibrillogenesis and mineral nucleation [19–22]. Loss of phosphate groups may occur during the natural extracellular enzymic processing and degradation of the protein following dentin mineralisation [10, 23–25]. Results of other studies have shown that this degraded protein has to be removed before remineralization can occur in completely demineralized samples of human dentin [26, 27]. This suggests that the "quality" of phosphophoryn, in terms of molecular form and structure, exerts a significant effect on mineral nucleation and crystal growth.

Protein-mineral binding is thus likely to play a central role in the biomineralization process. Wierzbicki et al. [28] using atomic force microscopy (AFM), observed proteins on calcite crystal surfaces. They described oyster shell protein as having a globular appearance on the crystal surface, whereas poly (Asp) was extended on the surface, giving it a layering effect. More recently the conformation of dentin phosphophoryn adsorbed to hydroxyapatite crystals using high-resolution solid-state nuclear magnetic resonance (NMR) spectroscopy was investigated [29]. It was concluded from this study that phosphophoryn may be an extended structure on the surface of those crystals and the β-sheet-like structure, favored by the molecular modeling studies [30], resembles locally the predicted structure of [Asp-Ser(P)-Ser (-P)]$_n$ [31].

It is probable that binding of proteins to developing hydroxyapatite crystals is governed by the interaction between specific protein motifs and charge domains on the crystal surfaces. Using chemical force microscopy (CFM) Kirkham et al. [32] have recently reported that the surfaces of developing enamel crystals carry a net positive charge, interrupted by small (~15 nm wide) areas of negative charge or lower positive charge density, perpendicular to the c-axis and separated by 30–50 nm along the c-axis. Such charge domains may therefore be candidate sites for matrix-mineral interactions.

In order to test this hypothesis, Kirkham et al. [32] investigated the possible relationship between the charge arrays on crystal surfaces and matrix-mineral binding using the AFM in fluid-tapping mode. These images revealed individual molecules or molecular aggregates for both serum albumin and amelogenin aligned on crystal surfaces, with spatial distributions correlating closely with the previously identified charge domains.

This system can also be used to provide a measure of protein-mineral binding by rinsing through the AFM fluid cell using phosphate buffers of increasing ionic strength to determine the point at which the proteins are desorbed [33, 34]. The data can be quantitated by measuring surface roughness of the crystals at all stages of the process.

The overall aim of the present study was to use the AFM in fluid tapping mode to investigate the interaction of dentin proteins with natural crystals of biological hydroxyapatite. Developing enamel crystals were used as a source of biological mineral for this purpose. The specific aims of this work were to

1. determine whether dentin protein-crystal binding was related to the charge domains recently described on developing enamel crystal surfaces;
2. compare the mineral binding capacity of DSP and phosphophoryn in order to determine whether the roles for these proteins in mineral nucleation and control of crystal growth are similar;
3. determine whether dephosphorylation of phosphophoryn affected its binding capacity

Methods

Proteins Used In Binding Experiments

Purified and biochemically characterized dentin phosphophoryn was obtained from immature tooth roots of human permanent teeth after demineralization in 10% EDTA. The resulting extract was desalted and DPP purified using $CaCl_2$ precipitation, DE4E-saphorose chromatographs, followed by $MgCl_2$ precipitation [35]. DSP was obtained, purified, and characterized from similar specimens using the method described by Butler et al. [36]. Dephosphorylation of phosphophoryn was carried out using alkaline phosphatase (ALP). Human phosphophoryn was dissolved in 0.1M Tris-HCl pH 8.0 and incubated with bovine intestinal ALP attached to agarose beads. The beads containing the enzyme were removed by centrifugation and the phosphate content was analyzed. It was found necessary to carry out the dephosphorylation twice to ensure its completeness [19]. Dentin proteins, obtained as described above, were dissolved in 20 mM Tris to a final concentration of 50 μg/ml. The pH of the solution was then adjusted to pH 7.4 by titration with small quantities of dilute HCl.

Enamel Crystals

Small particles (approximately 50-100μg in weight) of maturation stage enamel were micro-dissected free from the underlying dentin and used for the isolation of individual crystals. The enamel particles were first extracted with 0.1M sodium phosphrate buffer, pH 7.4 to desorb mineral bound and freely mobile protein species. The insoluble residue after extraction was pelleted by centrifugation and the supernatant removed. This was repeated for a total of 6 times. The pelleted material was then futher extracted in 50mM Tris containing 4M urea at neutral pH to dissolve aggregated protein. This extraction was also repeated 6 times with 0.1M phosphurate buffer, pH 7.4, to ensure final desorbtion of any mineral-bound components. The final extraction used distilled water with the pH adjusted to 7.0 in order to remove all traces of buffer and urea. Centrifugation and resuspension of the pellet was again repeated 6 times. Removal of protein from the crystals was monitored spectrophotometrically at 216nm and by polyacrylamide gel electrophoresis of the extraction solutions and following total dissolution of aliquots of the crystals using acetic acid [37–39].

Protein-crystal binding. The enamel crystals were sonicated for 2 min in HPLC-grade methanol to prevent aggregation [40]. Approximately 2 μl of this suspension was then placed onto freshly cleaved mica (Agar). The methanol was evaporated rapidly leaving a spread of dispersed hydroxyapatite crystals

on the surface. Fifty microliter of protein solution (phosphophoryn, DSP, or dephosphorylated phosphophoryn) for 120 sec was then added and the sample was introduced to the AFM fluid cell in the presence of approximately 100 µl of distilled water (pH 7.4). Crystals not treated with protein acted as controls.

All samples were imaged on a Digital Instruments Nanoscope IIIa Multimode AFM, equipped with an E-type scanner (~15 µm × 15 µm maximum scan range in x-y direction) using a commercially available fluid cell (Digital Instruments, Santa Barbara, CA). Tapping mode images were obtained using oxide sharpened silicon nitride NP-S cantilevers of quoted spring constant 0.32 N/m (Digital Instruments, UK Cambridge) and radii of 5–40 nm curvature. Cantilevers were resonated at approximately 9 kHz and images obtained at a tapping amplitude of approximately 80% of free amplitude.

Desorption of Protein from Crystal Surfaces

Desorption of proteins from the crystal surfaces was achieved using 200 mM phosphate buffer at pH 7.4. Time-lapsed images of the crystals were then obtained.

Measurement of Surface Roughness

Surface roughness measurements (R_a) were obtained over a length of 50–100 nm in an approximately central line parallel to the crystal c-axis. A minimum of 20 measurements were obtained in order to achieve a final mean roughness value. R_a roughness was calculated using the Nanoscope 4.23 software and is defined here as:

$$R_a = \frac{1}{L_x L_y} \int_0^{L_y} \int_0^{L_x} |f(x,y)| dx\, dy,$$

where $f(x,y)$ is the surface relative to the center plane and L_x and L_y are the dimensions of the surface. Therefore the calculated surface roughness in this case is the mean value of the surface relative to the center plane and takes into account any changes in the slope of the crystal surface.

Statistical analysis of the results of the roughness measurements for the protein-coated and non-protein crystal surfaces were analyzed using an unpaired Student's *t*-test.

Results

Figure 1 shows the image of the enamel crystals before (Fig 1a) and after (Fig 1b, c, & d) addition of the proteins. Figure 1b and c are images of the enamel crystal preparations following the addition of dephosphorylated phosphophoryn and DSP, respectively. Globular structures ("nanospheres" approximately 50 nm in diameter) were seen to be distributed on the mica and also in association with the crystal surface, but with no discernible pattern. Figure 1d shows the effect of adding phosphophoryn to the crystals. Phosphophoryn was also present on the surfaces of the mica and the crystals in the form of nanospheres similar in size and appearance to those seen for DSP and the dephosphorylated phosphophoryn. However, phosphophoryn binding gave rise to a distinctive banding pattern to the crystal surface.

The results of protein desorption experiments using 200 mM phosphate solution is seen in Figure 2. Figure 2a shows an image of the control enamel crystal preparation in the absence of proteins and treated with the phosphate buffer solution only. The smooth surface topography of the crystals can be seen. Time-lapsed images of the crystal surfaces after treatment with dephosphorylated phosphophoryn (Fig. 2b) and DSP (Fig. 2c) are shown after desorption with 200 mM phosphate solution. Protein nanospheres still appeared to be present on the mica surfaces but few, if any, were associated with the crystals which appeared very similar to the untreated controls. In contrast, Figure 2d shows the effect of phosphate desorption on the same crystals treated with phosphophoryn shown in Figure 1d. Distinct "banding" was again observed, apparently related to the retention of the phosphophoryn nanospheres on the enamel crystal surfaces.

Measurement of surface roughness of the crystals and crystal-protein preparations confirmed these observations, providing quantitative data, as shown in Table 1. Significant differences were observed in surface roughness following the addition of phosphophoryn to the crystals, which became rougher as a result. This increase in roughness was still present even after the addition of phosphate buffer. Addition of dephosphorylated phosphophoryn increased surface roughness in comparison with the crystals alone but this did not prove significant when tested. There was also no significant, effect when DSP was added to the crystals. Surface roughness after desorption of the dephosphorylated phosphophoryn, when compared to the crystal only control, decreased significantly.

Discussion

These AFM images are the first to show the interaction of dentin proteins from human teeth with naturally derived crystals of hydroxyapatite mineral. Ideally, imaging such interactions for phosphophoryn and DSP should be done using dentin crystals as the mineral source, but there are technical challenges that have yet to be overcome to permit this. Isolation of crystals from dentin without significantly altering their surface characteristics is extremely difficult due to the presence of the collagenous matrix within the tissue. In addition, the size of individual dentin crystals is extremely small, and anchoring them to substrates for scanning under fluid in the AFM remains a problem. For these reasons, crystals derived from developing enamel have been used in this study. These crystals are larger than those of dentin and offer the opportunity of using biological apatite to determine mineral-matrix associations, with the additional advantage of a previously characterized surface chemistry.

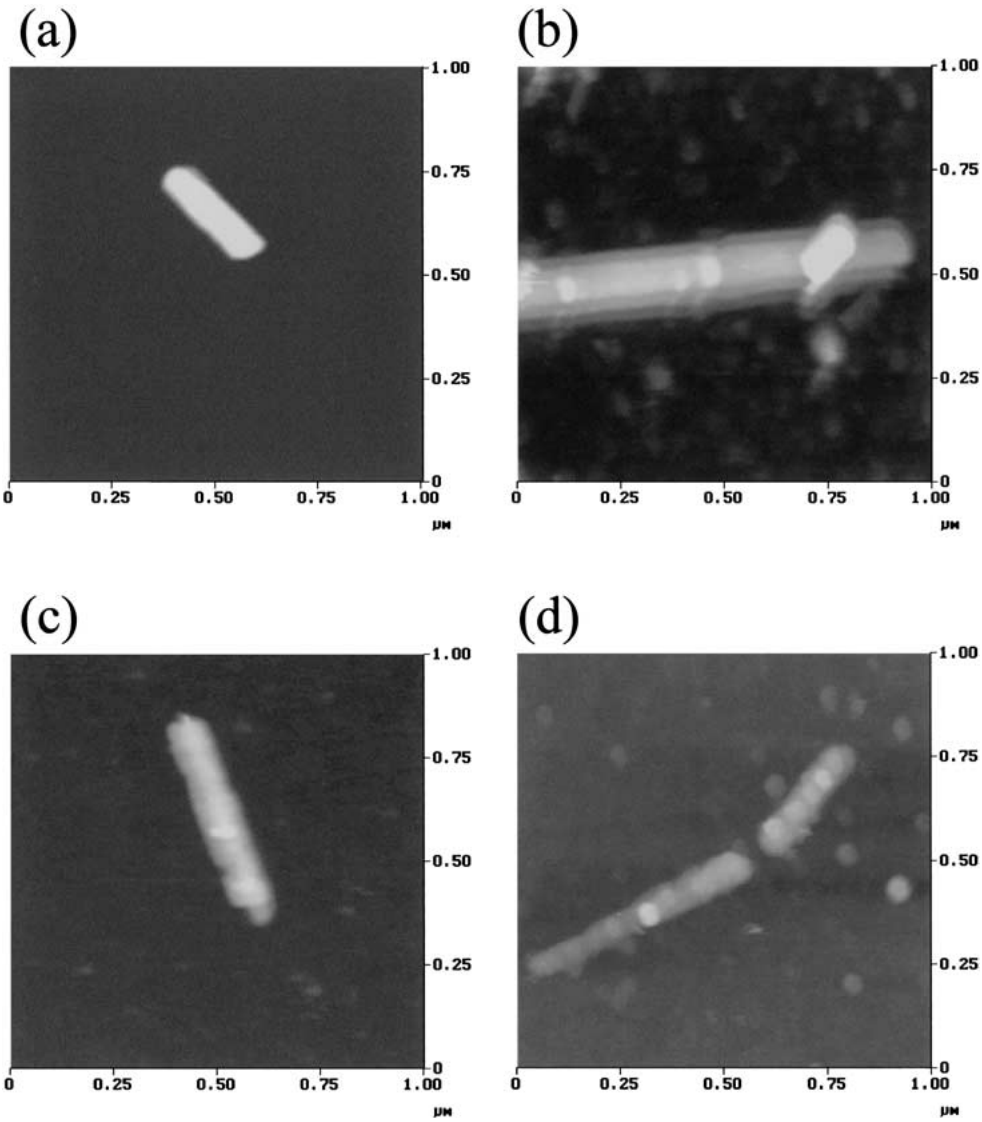
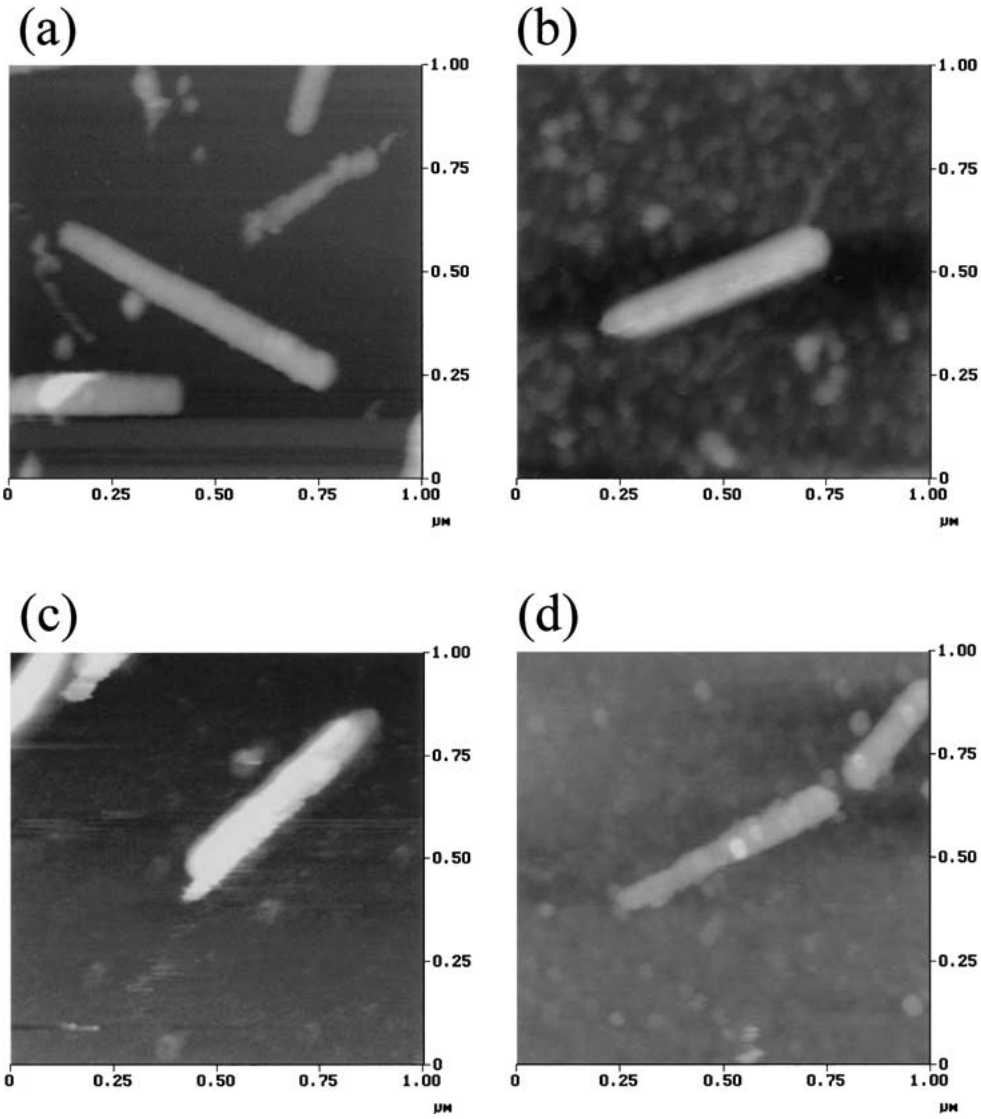


Fig. 1. Tapping mode AFM images of maturation stage enamel crystals before and after the binding of dephosphorylated phosphophoryn, DSP, and phosphophoryn, imaged under distilled water (pH 7.4). (**a**) Crystals imaged prior to the introduction of protein to the fluid cell. (**b**) Crystals imaged after the introduction of dephosphorylated phosphophoryn. (**c**) Crystals imaged after the introduction of DSP. (**d**) Crystals imaged after the introduction of phosphophoryn.

Our results clearly demonstrate protein-mineral association under conditions of physiological pH for phosphophoryn. There is less distinct evidence of such an association of dephosphorylated phosphophoryn and DSP.

In contrast to the theoretical description of hydroxyapatite crystals being covered by extended molecules of phosphophoryn [24], our observations would suggest that protein has a globular form not unlike that described for amelogenin [32] and for oyster shell protein [28]. Further, the globular structures observed in association with the crystal surfaces in the presence of the proteins investigated in this present study are similar in size to previous AFM studies of proteins, including BSA monomers [41, 42] and amelogenin aggregates [32]. At physiological pH, phosphophoryn would be expected to carry a high negative charge. Both dephosphorylated phosphophoryn and DSP would carry a much reduced negative charge in comparison. It is therefore probable that these proteins would all interact electrostatically with a net positively charged crystal surfaces. There is conflicting data in the literature as to whether hydroxyapatite surfaces have a positive or negative charge. Chander and Fuerstnau [43] measured zeta potentials and claimed that hydroxyapatite surfaces have both positive and negative charges. However, others have reported that the surfaces of biological hydroxyapatite crystals are positively charged possibly reflecting a calcium rich layer [40, 44].

The distribution of these proteins, especially phosphophoryn, on the crystal surface giving it a distinct



Tapping mode AFM images of maturation stage enamel crystals before and after the binding of dephosphorylated phosphophoryn, DSP, and phosphophoryn, imaged after the exchange of the solutionin the fluid cell with 200 mM phosphate buffer (pH 7.4) (**a**) Crystals imaged prior to the introduction of protein in buffer. (**b**) Crystals imaged after the introduction of dephosphorylated phosphophoryn and then buffer. **(c)** Crystals imaged after the introduction of DSP and then buffer. **(d)** Crystals imaged after the introduction of phosphophoryn and then buffer.

Table 1. Surface roughness (Ra) measurements of developing enamel crystals in the presence or absence of dentin matrix proteins and ± treatment with 200 mM phosphate buffer

Sample	Ra (nm) in water, pH 7.4	Ra (nm) in 200 mM phosphate buffer, pH 7.4
Crystal only (control)	0.78 ± 0.26 n = 50	0.78 ± 0.26 n = 50
Crystal + phosphophoryn	1.08 ± 0.42[a] n = 37	1.10 ± 0.33[a] n = 44
Crystal + dephosphorylated phosphophoryn	0.85 ± 0.27 n = 45	0.54 ± 0.15 n = 40
Crystal + DSP	0.79 ± 0.42 n = 59	0.72 ± 0.31 n = 42

Results show mean ± SD
[a] = significantly different from control ($P < 0.01$)

"banded" appearance is similar to that described for amelogenin [32] and for bovine serum albumin [42]. This banding appears most clearly on the upper crystal in Figure 1d, the phosphophoryn-treated specimen. Banding was apparent on other crystals treated with phosphophoryn and after desorption, but some of the resolution is lost in capturing the images. This concept, however, is supported in part by the roughness data. These bands seem to be of a similar periodocity to the charge arrays described by these authors using chemical force microscopy.

The results of the protein desorption experiments using 200 mM phosphate buffer would suggest that both the dephosphorylated phosphophoryn and DSP have less affinity for hydroxyapatite crystals than phosphophoryn. In fact, their distribution at the crystal surface may be a random event rather than any true binding to the enamel surface. The failure of the phosphate solution to desorb the phosphophoryn from the crystal surfaces would suggest that the interaction between phosphophoryn and the crystal surfaces is very strong. The surface roughness measurement, after the desorption of the dephosphorylated phosphophoryn, decreased significantly compared with the crystal only control (Table 1). Imaging in the presence of proteins is technically very challenging because of tip contamination which effectively decreases resolution. Thus, an explanation for the apparent smoother surface of this specimen is because of tip contamination when determining surface roughness. Again, the important comparator is with the protein-treated samples in the same experiment group, which in this case, are significantly rougher.

The high affinity of phosphophoryn for hydroxyapatite crystal surfaces certainly suggests a role for this protein in the control of crystal growth. These results also suggest that phosphorylation of phosphophoryn appears to be playing an important part in this "binding" to the crystal. This appears to be partially borne out by the work of Fujisawa et al. [20] who found that dephosphorylated phosphophoryn precipitated less calcium ions than the intact protein. The adsorption capacity of phosphophoryn onto hydroxyapatite was diminished by reducing the acidic groups due to dephosphorylation. This affinity may also be affected by the phosphophoryn concentration [45] and pH [46] both of which will affect intramolecular folding. There is less evidence to suggest that DSP would play such a role. It is less affinity for the crystal surfaces than phosphophoryn, and although dentin sialophosphoprotein (DSPP) is a unique molecule produced by a single gene, dspp, the ratio of DSP to phosphophoryn in dentin is 1:8–10, suggesting early degradation of this protein [47]. DSP has also been introduced into an *in vitro* mineralization system where it had a limited effect on apatite formation and growth. The authors also reported that DSP has a low affinity for hydroxyapatite seed crystals, which supports the AFM observations [48]. Certainly, other dentin matrix proteins, such as the proteoglycans, could be studied in the AFM using an enamel crystal protein desorption model.

In summary, although both DSP and phosphophoryn may promote mineral nucleation, it is less likely that DSP plays a continuing role in controlling subsequent crystal growth. However, phosphophoryn is a more likely candidate in controlling dentin crystal growth and morphology based on its high affinity for the crystal surfaces. This role is probably dependent upon its degree of phosphorylation, implying a mechanism for extracellular matrix modification in the control of biomineralization in skeletal tissues.

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