Amelogenin Induces Biomimetic Mineralization at Specific pH

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ABSTRACT

Amelogenin proteins are assumed to control the calcification of dental enamel with a nanoscale precision that facilitates the formation of fibrous apatite crystals organized in a remarkable microstucture. In this study, recombinant full-length human amelogenin induced protein-guided mineralization and the formation of an enamel-like composite material at specific physical-chemical conditions as observed by atomic force microscopy (AFM). Amelogenin bound specifically to fluoroapatite crystals (FAP) of a glass-ceramic substrate at Ca^{2+} and PO_4^{3-} concentrations similar to *in-vivo* conditions and at pH 8. Layers up to 400 nm high, containing elongated crystals, formed on the (001)-planes of FAP within 24h in supersaturated solutions. In contrast, (hk0)-faces grew by only 10-30 nm, but showed nanospheres aligned parallel to the *c*-axis of FAP. At pHs different from 8, proteins bound non-specifically to substrate and layers on FAP reached only 5-15 nm thickness. Micro-Raman spectroscopy and AFM revealed the formation of a composite material that resembled a structure and composition comparable to human enamel. These observations suggest that certain conditions are required to activate amelogenin to control and promote crystal growth of apatite along the *c*-axis and to synthesize an enamel-like material.

INTRODUCION

Enamel develops from a pure proteinacious tissue through complex interactions among organic, inorganic components and an aqueous fluid into a hard and durable tissue of 95 wt% mineral.[1-3] Ameloblasts express a series of structural proteins and proteases, of which at least 90 % are amelogenins, predominantly full-length peptides of 179 amino acids (25kDa).[1,4] The nascent molecule is bipolar, with a hydrophilic carboxyl terminus, but is hydrophobic over most of its length, containing only one phosphorylated site.[5,6] *In-vivo* and *in-vitro* investigations have revealed some putative functions of amelogenins.[2,7] Amelogenin inhibits apatite nucleation[8-10], but appears to direct crystal growth almost exclusively in the *c*-axis direction. In a current model, amelogenin nanospheres adhere preferentially to the (hk0)-faces of the apatite crystals, blocking these crystal planes from further growth.[1,2] Crystals are forced to grow into the spaces provided by the proteins, favoring crystal elongation along the *c*-axis and preventing early crystal fusion. The carbonated apatite crystals of enamel are only 50 nm thick, but several hundred micrometers long. They are covered by a thin protein layer and organized in a textured microstructure.[11] Understanding the mechanisms that control organic and inorganic

molecular interactions may allow the use of such biomolecules for the synthesis of composites with structural control on the nanometer level mimicking the natural tissue.

MATERIALS AND METHODS

In this study, human recombinant full-length amelogenin was used for the first time (rH174) in *in-vitro* biomineralization experiments at concentrations (0.4 mg/ml) that exceeded earlier experiments[9,10,12,13] and in an ionic environment similar to the fluid of the developing enamel.[14] An aluminophosphosilica glass containing oriented rod-like FAP crystals 1 µm wide and 3-6 µm long served as a substrate for the mineralization experiments.[15] The FAP crystals were aligned parallel to their *c*-axes by extruding crystallized glass melts at 1200ºC, as described in the literature.[16] Polishing sections perpendicular or parallel to the extrusion axis provided substrates that exposed either predominantly hexagonal (001)-planes, (Fig. 1a), or rectangular (hk0)-planes, respectively. Fig. 1a shows that crystal surfaces (dark) appear 2-6 nm below the glass level (bright). These substrates are advantageous, because they are flat enough to image assembled proteins, similar to glass or mica substrates,[17,18] but also allow in-situ studies on *in-vitro* biomineralization, since apatite is the mineral phase of calcified tissues of vertebrates. The height difference between glass matrix and the FAP crystals served as a reference to precisely measure crystal growth. Polished substrates were immersed into a) buffered solutions containing 0.4 mg/ml of the recombinant protein rH174, b) supersaturated solutions containing 0.5 mmol/l Ca^{2+} and 2.5 mmol/l PO_4^{3-} (CaP-sol), as found in the developing enamel,[14] without proteins at various pH-values, or c) CaP-sol at various pH containing rH174 at concentrations of 0.4 mg/ml. Recombinant proteins, rH174, were obtained by cloning and expressed by *E.coli* bacteria as described previously[19] and in materials and methods. The *in-vitro* mineralization experiments were performed in 1.5 ml siliconized test-tubes at pH-values of 6.4, 7.3, 8.0 and 8.8 at 37°C for up to 24h as described in materials and methods. Micro-Raman spectroscopy, (spectrometer HR 800, Jobin Ivon, Horiba Group) was used with a 20mW HeNe-Laser at a wavelength of 632.8 nm for determination of calcium phosphate phases and potential incorporation of an organic phase.

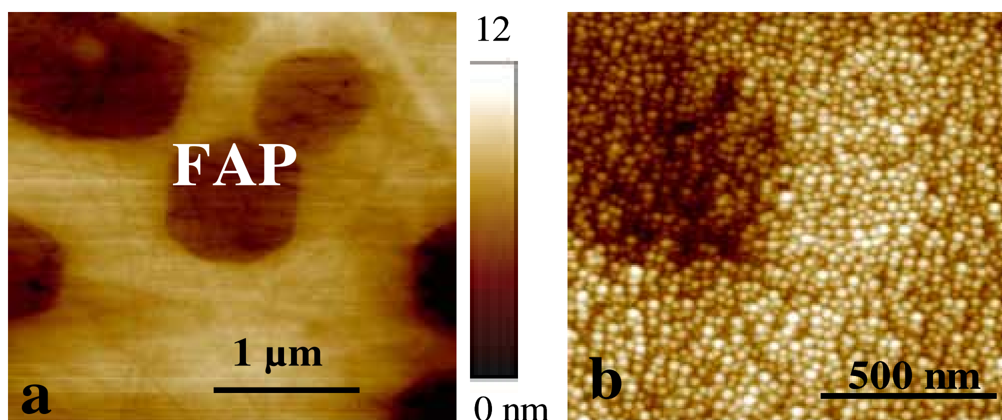

Figure 1: AFM-images of FAP glass-ceramic substrates a) hexagonal (001)-planes of FAP after polishing before experiment; b) after 24h immersed into amelogenin solution (0.6 mg/ml), protein binds nonspecifically to substrate at pH 7.3.

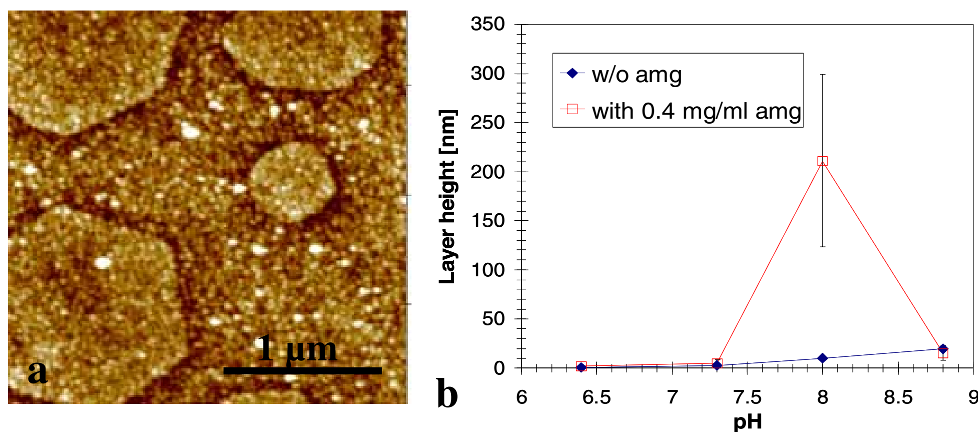

Figure 2. a) AFM-image of (001)-plane of FAP after immersion into protein-free CaP-sol. In 24 h, FAP crystal grew about 15 nm above the glass level. b) Height of layer measured as step height between glass and layer on (001) planes of FAP after 24 h of mineralization without and with amelogenin (0.4 mg/ml). When proteins were present layer height increased drastically at pH 8.

RESULTS AND DISCUSSION

Immersion into protein only or CaP-sol only: The topography of the glass-ceramic substrate before immersion is shown in the AFM image of Fig. 1a. Substrates were immersed into calcium-free buffered solutions containing 0.4mg/ml rH174 at pH from 6.4 to 8.8. Amelogenin nanospheres with diameters around 20 nm[1] adhered not only to the FAP crystals, but also to the glass matrix as revealed by tapping mode AFM imaging in Fig. 1b. When glass-ceramic substrates were immersed into CaP-sol without proteins, mineral precipitated as 15-20 nm grains (Fig. 2a), and the original FAP crystal increased in height. At 5h immersion and a pH of 8, glass and FAP crystals reached approximately the same height level. After 24h the apatite on the FAP crystal grew about 15 nm above the glass level (Fig. 2a), as a result of isoepitaxial growth. Fig. 2b shows that the final layer height at 24h increased with pH (and supersaturation) of the solution.

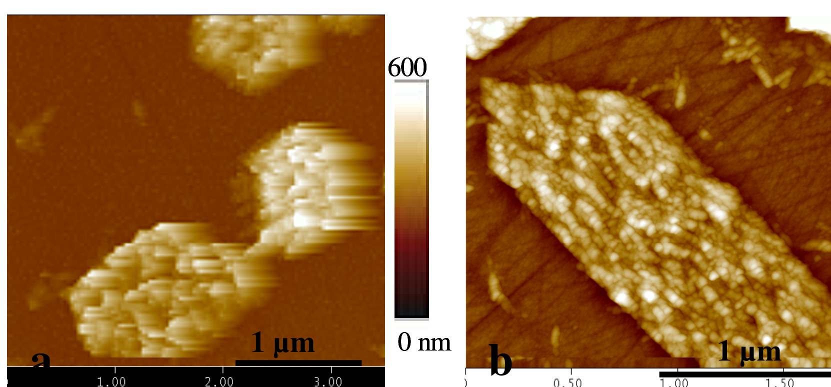

Figure 3: AFM-images of layers grown in CaP-sol containing 0.4mg/ml rH174 a) On the (001)-plane of FAP at pH 8.0, a layer of 300 nm height formed specifically on FAP and revealed oriented elongated crystals, b) In the layer grown at pH 8 on (hk0)-plane of FAP, nanoparticles on (hk0)-plane arrange in short string-like patterns approximately parallel to the *c*-axis of the underlying FAP crystal. Few or no amelogenin nanospheres or mineral precipitates were observed on the surrounding glass.

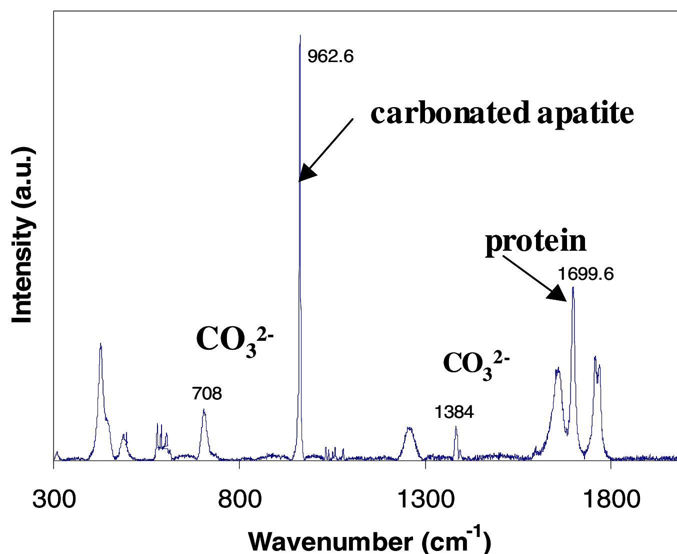

Figure 4.: Micro-Raman spectroscopy of biomineralized layer at pH 8, 24 h, revealing the formation of a biocomposite consisting of carbonated apatite and proteins.

Immersion into CaP-sol containing 0.4 mg/ml protein: When amelogenin protein, rH174, was added to the calcium phosphate solutions at pH between 6.4 and 8.8, to obtain a final concentration of 0.4 mg/ml, no significant differences in the crystallization behavior and final layer height compared to the protein-free calcium phosphate solutions were observed at any pH values except pH 8, as shown in Fig. 2b and in the AFM image of Fig. 3a. At pH 8 a strong increase in the height of layers formed on FAP was observed (Fig. 2b). Crystals grew up to 400 nm above the glass level, as shown in Fig. 3a. The hexagonal shape of the original (001)-plane was maintained after crystallization. AFM imaging revealed that the grown layers consisted of needle-like structures pointing away from the surface in parallel alignment (Fig. 3a). In contrast to the experiments done at other pH values, at pH 8 and in the presence of Ca^{2+} and PO_4^{3-} ions, amelogenin-nanospheres did not adhere to the glass matrix. As shown in Figs. 3a and b, the reaction with the solution was restricted to the area of the FAP crystal. Little or no precipitate and few protein nanospheres were observed on the glass.

Interaction of proteins with (hk0)-planes of FAP: Glass-ceramic substrates that exposed predominantly (hk0)-surfaces were immersed into CaP-sol containing 0.4mg/ml rH174. In agreement with observations on (001)-planes, growth patterns were altered on (hk0)-planes only a pH around 8. The final layer height at 24 h was slightly increased (10-30 nm), but at least 3 to 4 times smaller than on (001)-planes (100-400 nm), as shown in Fig. 3b. Layers grown on (hk0)-planes at pH 8, however, were textured. As shown in Fig. 3b, strings of 4 to 8 nanospheres (diameter = 40-60 nm) were aligned parallel to each other along the *c*-axis of the underlying FAP crystals. Amelogenin also adhered specifically onto the (hk0)-planes of FAP at pH 8, while it adhered non-specifically to the substrate at other pH-values, in agreement with the observations on (001)-orientation.

Micro-Raman spectroscopy on these samples showed the formation of apatite in the mineralization experiments, as revealed by the strong band at 962 cm^{-1} in Fig. 4a.[20] The spectrum also exhibited bands at 708 and 1384 cm^{-1}, indicating that CO_3^{2-} ions were incorporated into the apatite lattice. Furthermore, bands around 1700 cm^{-1} were observed and attributed to bending modes of N-H groups of proteins.[20] Hence, a composite material comprised of protein and mineral has formed. Protein incorporation was not detected on samples that were immersed into amelogenin containing CaP-sol at pH different from 8.

DISCUSSION

A glass-ceramic substrate was used to study the influence of pH on the *in-vitro* biomineralization potential and binding affinity of recombinant human full-length amelogenin, rH174, in the presence of Ca^{2+} and PO_4^{3-} ions at concentrations found in-vivo. The experiments suggest that a pH around 8 is required to activate recombinant full-length human amelogenin to specifically bind to apatite and accelerate apatite growth on (001)-surfaces of FAP, inducing the formation of a composite comprised of mineral and protein.

The formation of thin layers on FAP crystals of the substrate was not significantly altered by the presence of amelogenin and presumably amelogenin was not incorporated into the newly formed layer at pH-values of 6.4, 7.3 and 8.8. In contrast, at pH 8.0, amelogenin strongly interacted with apatite. The layers formed specifically on the FAP crystals of the substrate and were increased up to 20 times in height (see Fig 2b). Amelogenin-nanospheres did not adhere to the glass at this pH. Compared to the protein-free experiments, mineral layers grew about 20 times faster when amelogenin was present. The increased crystallization rate, however, was only observed on (001)-planes of FAP. When crystal faces perpendicular to the *c*-axis of apatite were presented to the protein, binding was FAP specific, but no significant increase in layer height was observed. In agreement with current models, it is therefore assumed that amelogenin prevents crystal growth in the lateral dimension of fibrous apatite crystals.[1,2] The pH dependent functionality of proteins and enzymes is commonly associated with a change in tertiary structure that results in a higher specificity and activity of the protein. Comparable to observations made on phosphophoryn, we assume that changes in the tertiary structure of amelogenin cause increased affinity to apatite and activate the protein to guide the mineralization process.[21] This hypothesis will be further investigated by detailed structural analysis of amelogenin at various pH.

CONCLUSIONS

The experiments suggest that a pH around 8 and the presence of Ca^{2+} and PO_4^{3-} ions is required to activate recombinant full-length human amelogenin to specifically bind to apatite and promote and accelerate apatite growth on (001)-surfaces of FAP, generating an enamel-like material. Understanding the mechanisms behind protein-guided mineralization may provide us with the tools to design materials with structural control on the molecular level.

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