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Control of Calcium Phosphate Nucleation and Transformation through Interactions of Enamelin and Amelogenin Exhibits the “Goldilocks Effect”

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Abstract

Although amelogenin comprises the vast majority of the matrix that templates calcium phosphate nucleation during enamel formation, other proteins, particularly enamelins, are also known to play an important role in the formation of enamel’s intricate architecture. However, there is little understanding of the interplay between amelogenin and enamelins in controlling processes of mineral nucleation and growth. Here, we used an *in vitro* model to investigate the impact of enamelins interaction with amelogenin on calcium phosphate nucleation for a range of enamelins-to-amelogenin ratios. We found that amelogenin alone is a weak promoter of nucleation, but addition of enamelins enhanced nucleation rates in a highly nonlinear, nonmonotonic manner reaching a sharp maximum at a ratio of 1:50 enamelins/amelogenin. We provide a phenomenological model to explain this effect that assumes only isolated enamelins proteins can act as sites of enhanced nucleation, while enamelins oligomers cannot. Even when interaction is random, the model reproduces the observed behavior, suggesting a simple means to tightly control the timing and extent of nucleation and phase transformation by amelogenin and enamelins.

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ASSOCIATED CONTENT

Supporting Information

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AFM images of amelogenin–enamelins adsorption, AFM images of calcium phosphate nucleation, SEM images of mineral morphology with amelogenin rP172, TEM images of minerals formed at different stages of nucleation, schematic illustrating the liquid cell for Raman spectroscopy, and curves representing the equilibrium coverage of hypothetical enamelins monomers as represented by the green dots in Figure 4 (PDF)

The authors declare no competing financial interest.

Graphical Abstract

INTRODUCTION

During mineralization of hard tissues, soluble proteins act in a conjunction with an insoluble macromolecular matrix to guide biomineral formation.¹ For example, recent work demonstrated that, in the case of calcite forming coccolithophorids, this cooperative effect is so important that, in the absence of the soluble protein, no mineralization of the matrix occurs.² In some cases, such as tooth enamel, the matrix, which comprises primarily amelogenin, is processed by enzymes after secretion into the extracellular environment so that the end product contains only trace amounts of the original matrix.³ In other tissues, like bone and dentin, the collagen-based insoluble matrix becomes an essential part of the resulting structure and contributes to tissue function.⁴ Bone, dentin, and enamel all contain numerous matrix–mineral interfaces during their development. For example, nonamelogenins in enamel or noncollagenous proteins in dentin and bone commonly have specific motifs that coassemble with the matrix proteins amelogenin and collagen and additional charged functional groups that interact with the mineral surfaces.^{5–10} One of the biggest challenges in understanding biomineralization is to reveal the structural and functional relationship between the mineralizing constituents, the insoluble matrix, and the soluble proteins during the different stages of mineral nucleation, phase transformation, and growth.

Amelogenin is considered necessary for proper formation of dental enamel, as it comprises more than 90% of the organic component in the early stage of enamel formation.¹¹

Amelogenin protein is mostly hydrophobic and contains a hydrophilic C terminus. It self-assembles *in vitro* into nanospheres or nanoribbons depending upon the pH and the calcium or phosphate species concentration in solution.^{12–18} Amelogenin-null mouse models demonstrate the dramatic importance of amelogenin, as they exhibit a type of amelogenesis imperfecta (AI) with disrupted patterns of enamel rods that compromise the integrity of the enamel.^{19,20} Moreover, amelogenin has been found to mediate calcium phosphate nucleation and growth *in vitro* in different experimental settings.^{18,21–23}

Despite the fact that amelogenin comprises the vast majority of the enamel-forming matrix, it does not act alone. Nonamelogenin proteins like enamelin and ameloblastin play crucial

roles in enamel formation, even though they are present in smaller quantities. For example, enamelin, a large glycoprotein (Mw = 186 kDa), constitutes <5% of the extracellular matrix but is required for enamel mineral formation, as shown unequivocally in gene-targeted mouse models and human mutations resulting in hypoplastic AI.^{24–28} No true enamel or apatite mineral ribbons are formed in *Enam*^{−/−} mice.²⁹ The abnormally thin and disorganized enamel in AI patients suggests that enamelin is required to drive crystal formation, achieve structural organization of the apatite prisms, and develop optimal enamel thickness.^{26,27} Despite its critical function in normal enamel formation, the mechanistic role of enamelin in the development of the mineral is not yet understood.

Like amelogenin, enamelin is processed by enamel proteinases immediately upon secretion. The major and the most stable cleavage product, 32 kDa enamelin, distributes throughout the developing enamel layer.^{30–33} Importantly, some mutations in the enamelin gene causing AI have been reported to be within the 32 kDa enamelin fragment.³⁴ *in vitro* studies have demonstrated that the interaction between amelogenin and enamelin has a structural impact on both proteins, as well as a synergistic effect on apatite nucleation and octacalcium phosphate (OCP) crystal morphology.^{35–37} In the present study, to advance understanding of the interplay between amelogenin and enamelin in controlling enamel mineralization, we used *in situ* atomic force microscopy (AFM), *in situ* Raman spectroscopy, scanning (SEM) and transmission electron microscopy (TEM), and X-ray scattering to evaluate the effect of amelogenin and the 32 kDa enamelin interactions on the nucleation, growth, and phase transformation of calcium phosphate *in vitro*. We followed nucleation and growth events in bulk solution as well as on substrates with different degrees of hydrophobicity. Finally, we utilized Grand Canonical Monte Carlo (GCMC) simulations to elucidate how coassembly impacts the status of enamelin monomers and aggregates, and to provide a rationale for the observed cooperative and dose-dependent effects.

RESULTS AND DISCUSSION

First, we used *in situ* AFM to observe the coassembly of purified recombinant porcine full-length amelogenin rP172 (19.6 kDa) and native 32 kDa enamelin fragments on muscovite mica for a range of enamelin/rP172 ratios, following the sample preparation protocol shown schematically in Figure 1. Phosphate solutions containing amelogenin and enamelin were injected into an AFM fluid cell, and the protein coverage was determined as a function of time without flow (Figures 2a,b and S1). The results show that increasing the enamelin/rP172 ratio led to slower adsorption kinetics (Figures 2c and S1) with a smooth decrease in adsorption rate coefficient (Figure 2d). Previous *in vitro* biophysical studies performed in solution have demonstrated that 32 kDa enamelin had the effect of dissociating amelogenin nanospheres that formed at pH 8, whereas it stabilized smaller amelogenin oligomers.⁵

Moreover, we have shown that amelogenin nanospheres dissociate and are not stable on charged surfaces.³⁸ The addition of enamelin to amelogenin may affect the charge distribution on the protein particles formed in the solution, hence resulting in their different adsorption behaviors.^{5,30,31} The height distribution of particles did not change at different enamelin/rP172 ratios, which indicates that enamelin does not change the association between rP172 assemblies when adsorbed on the surface (Figure S1g). The slower

adsorption kinetics of enamel/rP172 complexes at higher enamel/rP172 ratio may be therefore due to the stronger repulsive force between negatively charged mica and negative charged enamel/rP172 complex.

After mixing amelogenin and enamel in phosphate solution, the AFM fluid cell was flushed with water to remove the phosphate buffer. Mineralization of the protein-covered surfaces was then induced by flowing supersaturated calcium phosphate solution containing 1.5 mM CaCl_2 and 9.5 mM Na_2HPO_4 (pH 7.40) into the fluid cell (Figure 3a–d). As previously shown, under these conditions, the initial mineral phase formed was amorphous calcium phosphate (ACP), which then transformed into octacalcium phosphate (OCP) and finally to HAP.³⁹ The results show that the inclusion of enamel impacted the rate of ACP nucleation (Figure 3e,f), but the effect was highly nonlinear, exhibiting a sharp maximum at an enamel/rP172 ratio of 1:50, when the rate was enhanced by 400% over the value of amelogenin-adsorbed films in the absence of enamel. In contrast, when the enamel/rP172 ratio was increased above 1:50, nucleation was inhibited (Figure 3f). The sharp maximum at an enamel/rP172 ratio of 1:50 is achieved in repeated experiments and exceeds the rate for pure amelogenin by five to 15 times the error in the measurements. This dose-dependent effect on calcium phosphate nucleation in which a sharp maximum was reached at a low enamel concentration and was then followed by inhibition was observed on the surfaces of highly ordered pyrolytic graphite (HOPG) (Figure S2). Such a surface is both uncharged and highly hydrophobic, showing that neither the surface charge of mica nor hydrophobicity of HOPG affected the observed dose-dependent effect.⁴⁰

To determine whether the effect of enamel on ACP nucleation when added with rP172 was influenced by some other property of the substrates, we monitored the separate and combined effects of these two proteins on calcium phosphate nucleation and phase transformation in bulk solution and followed the progress of nucleation by *in situ* Raman spectroscopy and X-ray diffraction (Figure 4a–f). Based on the position of the Raman peak (Figure 4c) and the short-range order within 0.5 nm in the X-ray pair distribution function (PDF) (Figure 4f), we conclude that, in the absence of protein, the mineral formed was still amorphous even after 30 min. By about 40 min, long-range order up to 1.5 nm became evident in the X-ray PDF data, and the Raman peak shifted to the position characteristic of crystal, which indicates the phase transformation started at 40–50 min. Short, rod-shaped crystals were observed in TEM images around the surface of ACP particles when the phase transformation began, and the number and length of these short crystals increased with prolonged incubation (data not shown). Finally, the crystals were in the shape of thin plates with less controlled crystal orientation, as shown by both morphology and electron diffraction (Figures S3 and S4). The presence of the proteins individually or in combination does not appear to have a strong effect on the crystal morphology after 200 min though the kinetics of transformation are altered (Figure S4).

The time evolution of the optical images in the presence of protein shows that rP172 alone enhanced the rate of ACP nucleation (Figures 4a and S5) while also increasing the lifetime of the ACP phase (Figure 4d,e). However, when rP172 and enamel were mixed together in solution, a more complicated dependence on enamel/rP172 ratio was observed; the nucleation rate again increased with enamel/rP172 ratio, reaching a maximum at ~4:50

(Figure 4a,b). The lifetime of the ACP phase (Figure 4d,e) also increased with increasing enamel/rP172 ratio, reaching a maximum at a ratio of about 2:50, at which the lifetime exceeded that observed with rP172 alone. However, the lifetime then decreased to a minimum at about 8:50; at this ratio, the lifetime was below that obtained with either rP172 or enamel alone (Figure 6S), before increasing again to a level equivalent to that of rP172 alone (Figure 4e).

The results show that, over the range of enamel/rP172 ratios in which nucleation of ACP is enhanced by the presence of enamel, the degree to which nucleation of the more stable phase is inhibited in bulk solution is also increased, and thus, ACP can survive for longer times over this range. Moreover, these results show that the synergistic effect of enamel and amelogenin occurred both on substrates and in bulk solution. This synergistic and dose-dependent effect has also been reported for the nucleation of apatite crystals inside a gelatin gel diffusion system.³¹ Similarly synergistic regulation by 32 kDa enamel and a recombinant pig amelogenin affected the morphology of (OCP) crystals,³⁷ with the interplay of enamel and amelogenin enhancing the stability of the transient amorphous calcium phosphate (ACP) phase and increasing the length-to-width ratio (aspect ratio) of the OCP crystals.³⁷

These results demonstrate that enamel is a promoter of ACP nucleation when coassembled with amelogenin regardless of whether it is in bulk solution or deposited on a surface, provided the concentration is sufficiently low, with the degree of enhancement exhibiting a sharp maximum near an enamel/rP172 ratio of 1:50 on a surface and 4:50 in bulk solution. It is noteworthy that both proteins, enamel and amelogenin, stabilize ACP phase, but their ability to stabilize ACP is maximized in a similar range of enamel/amelogenin ratios (1:50–4:50) at which nucleation is most significantly enhanced, both in solution and when adsorbed on surfaces.

The fact that the degree of ACP nucleation enhancement decreases above a certain ratio implies that the observed dependence is not simply a result of saturating the available surface sites (i.e., above a certain concentration, no further gain is achieved). Rather, the dependence implies that, above a certain concentration of enamel, further addition of enamel *eliminates* the sites of enhancement created at lower concentration. To explain this effect, we propose a simple statistical model that hypothesizes isolated enamel monomers are sites of enhanced nucleation, but those that have other enamel monomers as neighbors will form aggregates or oligomers of enamel whose conformation or exposed surface differs from that of the isolated proteins and renders them unable to promote nucleation. This assumption is based on the previous reports that support the calcium-binding potential of 32 kDa enamel.³⁵ Circular dichroism spectroscopy showed that enamel exhibits a conformational change with a preference for the β -sheet upon addition of calcium ions.³⁵ In addition, previous studies using DLS showed that the 32 kDa enamel solution is monomeric up to 6.4 $\mu\text{g/mL}$ in neutral pH bulk solutions with calcium ions, but above this concentration, large aggregates were detected.⁵ This indicates that the 32 kDa enamel has a strong self-binding nature and forms large aggregates when it reaches a critical concentration.

To determine whether this hypothesis leads to a concentration dependence of nucleation promoters that is consistent with the observed dependence, we used a Grand Canonical Monte Carlo algorithm^{41,42} to simulate the random deposition of monomeric adsorbates onto a surface and analyzed the results to determine the number of isolated “monomers” on that surface as a function of the total fraction of surface sites containing an adsorbate (Figure 5a,b). The results show that the percentage of surface sites containing an isolated monomer is a strong, nonlinear function of adsorbate coverage (Figure 5c) that rises linearly at low coverage and reaches a sharp peak at a fractional coverage of about 0.02, before falling rapidly. Equating the adsorbates (green squares in Figure 5b) to enamel and the open sites (white squares in Figure 5b) to rP172, the maximum then lies near an enamel/rP172 weight ratio of 0.022, which corresponds to an enamel/rP172 ratio close to 1:50 in bulk solution (Figure 5d). Above that ratio, the fraction of isolated monomers falls rapidly, while the percentage that has one or more neighbors rises. Introducing an attractive interaction between nearest neighbors biases the adsorption maximum peak toward a lower enamel/rP172 ratio as enamel self-aggregates more readily,⁵ whereas introducing a repulsive interaction has the opposite effect (Figure S7). However, the maximum peak still always exists in these cases.

With the results of this simple statistical model, the dependence of the nucleation rate on enamel/rP172 ratio can be understood, provided isolated enamel proteins within the amelogenin matrix are preferred sites of nucleation, while those with enamel neighbors (i.e., enamel oligomers or aggregates) are inactive. As the enamel/rP172 ratio increases, at first the number of isolated enamel sites increases rapidly, and thus, the rate of nucleation also increases rapidly. However, beyond the value where the percentage of enamel sites that are isolated reaches a maximum, which is expected to be in the vicinity of 1:50, the rate of nucleation begins to drop again. Because active nucleating sites that would have been covered with amelogenin are now covered with enamel in the form of aggregates or oligomers, the rate of nucleation drops below that of a pure amelogenin film, leading to a regime of inhibition, as observed experimentally (Figure 3f).

These findings raise the question of why enamel monomers would promote nucleation, but enamel aggregates or oligomers would not. Generally speaking, protein aggregation and misfolding can be expected to interrupt the structured assembly needed to promote ion binding and mineral nucleation. Structured assembly of proteins is the basis for directed mineral nucleation, both in natural biominerals and in biomimetic systems,^{43–45} and can easily be understood as a result of the local structural and chemical environment created through assembly. One clear example is the case of silica production by silicateins, during which distinct peptide residues are brought into juxtaposition.⁴⁴ The loss of protein function in inducing mineral nucleation can also be explained if the protein–protein interactions leading to random aggregation or structured oligomerization involve the peptide segments or side chains that are required for promotion of mineral nucleation. Because we do not know which structural and/or chemical features of enamel are responsible for promotion of ACP nucleation, we cannot identify which enamel–enamel interaction motifs are responsible for the loss of function. Nonetheless, given that 32 kDa enamel is highly charged,^{30,31} as are the regions often implicated in promotion of mineral nucleation by proteins and peptides,^{43–45} blocking the exposure of charged side chains is one obvious explanation for the

observed effect. We speculate that during enamelin oligomerization or aggregation these highly charged anionic or cationic regions may undergo coordination with one another via Ca^{2+} or HPO_4^{2-} species and thus no longer be available to promote nucleation.

In summary, the *in vitro* experimental results presented here have important implications for the functional mechanisms of proteins in enamel mineral formation. While the bulk solution concentrations of proteins used in these experiments are below the *in vivo* protein concentrations in enamel fluid, we believe that the physiochemical events that occur at the mineral–protein interface may well be relevant. The *in vitro* models used here are designed to follow such events at high precision and at molecular or atomic levels, and the surfaces employed act to enhance protein concentrations well beyond their bulk values. Thus, they may well mimic the *in vivo* situation at the protein–mineral interface. Moreover, whether the protein concentrations at the time of mineral genesis in these isolated environments are at the elevated levels seen in enamel fluid, or are substantially smaller, is unknown. Finally, the principle elucidated by these results, that a tendency toward colocalization by proteins can lead to complex dependencies of mineral formation kinetics on relative concentrations simply due to the statistics of interactions, is a general one that is applicable regardless of the specific proteins under consideration or their concentrations.

Our results support the concepts of (a) protein dose dependency, (b) cooperative effects between proteins, and (c) the control of calcium phosphate phase transformation in enamel formation. The 32 kDa enamelin selected for this study is the most stable cleavage product of the full-length enamelin and the only one that can be purified and isolated. It is rich in glutamic and aspartic acid residues and is highly phosphorylated.^{30,31} It exhibits a remarkable level of control over nucleation and phase transformation kinetics, as well as final morphology.^{29,37} Alteration of enamelin phosphorylation sites leads to enamel malformation in AI patients.⁴⁶ The present study highlights the concept of enamelin dose dependency, which has also been demonstrated in transgenic mouse models in which expression levels of the enamelin gene were controlled. Hu et al.²⁹ have shown that only an optimal quantity of enamelin will lead to normal enamel formation. Deficiency or excess of enamelin protein eradicates the formation of enamel crystals and affects prism formation.²⁷ The concept of synergy between enamel-forming proteins was put forth in an *in vivo* study of double knockout mice in which the genes for amelogenin (*Amel*) and ameloblastin (*Ambn*) were deleted or mutated.⁴⁷ The destructive effect on enamel was more severe in the double knockout compared to *Amel* or *Ambn* single knockouts.

The results of the present study provide additional *in vitro* evidence for cooperative effects between amelogenin and enamelin. We have previously reported interactions and colocalization between amelogenin and enamelin in enamel.^{5,6} We have further demonstrated their cooperative function in controlling crystal morphology.³⁷ Here, we report the precise level of control over ACP nucleation and phase transformation by these two proteins. The fact that these two proteins interact and colocalize during the early stage of enamel formation supports the notion that the stability of the ACP mineral phase at the early state of enamel formation is not merely controlled by amelogenin and its proteolytic products,^{48,49} but it is a cooperative function between amelogenin and nonamelogenins. Given the large diversity of enamel proteins including enamelin, ameloblastin, and amelotin,

and considering the complexity in their pattern of expression, secretion, processing, and assembly, the research presented here provides a general strategy to evaluate the synergetic role of these proteins through coassembly in controlling the mineralization process. Understanding such molecular processes is a key step enabling the synthesis of biomimetic hybrid materials for medical applications.

EXPERIMENTAL PROCEDURES

Amelogenin Expression and Purification.

Recombinant porcine amelogenin (rP172) was expressed in *Escherichia coli* strain BL21-codon plus (DE3-RP, Agilent Technologies, Inc., Santa Clara, CA) as previously described.^{50–52} Protein purification was accomplished on a reverse phase C4 column (10 × 250 mm, 5 μm) mounted on a Varian Prostar HPLC system (ProStar/Dynamics6, version 6.41 Varian, Palo Alto, CA), using a linear gradient of 60% acetonitrile at flow rate of 1.5 mL/min. Recombinant porcine amelogenin rP172 is an analog of full-length native porcine P173, which has 173 amino acids, but rP172 lacks the N-terminal Met and a phosphate group on Ser16.⁵³

Enamelin Isolation and Purification.

The 32 kDa enamelins were extracted from unerupted second and third mandibular molars of six-month-old pig jaws, purified and characterized as described previously following the method described by Yamakoshi et al.^{5,30,31} Samples were scraped from second and third unerupted molars (Farmers John Clougherty Co., Los Angeles, CA, USA, through Sierra for Medical Sciences, Santa Fe Springs, CA, USA). The pooled enamel samples were homogenized in 50 mM Sørensen buffer (pH 7.4) with proteinase and phosphatase inhibitors. This extraction process was repeated three times. The combined supernatant was processed and purified by reverse-phase high performance liquid chromatography (RP-HPLC) as reported previously.³⁵ The purified 32 kDa enamelins were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Stains-All staining, and Edman degradation.³⁵

Amelogenin rP172 and Enamelin 32 kDa Coassembly.

The rP172 and enamelins 32 kDa were dissolved separately in pure water to yield final concentrations of 1000 and 300 μg/mL, respectively. The rP172 was diluted in a phosphate buffer containing 19 mM Na₂HPO₄ at pH 7.40. The final rP172 concentration was fixed at 50 μg/mL in all cases. Enamelins 32 kDa were introduced into rP172 solution at various final enamelins concentrations of 1 μg/mL (1:50), 2 μg/mL (2:50), 4 μg/mL (4:50), and 8 μg/mL (8:50). Then the mixed protein solutions were injected into liquid cells with a freshly cleaved muscovite mica disc (diameter 9.9 mm, Ted Pella, Inc.) or highly oriented pyrolytic graphite (HOPG) (10¹⁰ mm, Ted Pella, Inc.) on the bottom, the solution was incubated in the liquid cell, and the adsorbed proteins were imaged by *in situ* AFM, with the nonadsorbed proteins flushed away by water after 50 min. The images were analyzed using the image processing software package SPIP 5.1.4 (Image Metrology A/S, Hørsholm, Denmark). The particle size distribution and areal coverage were calculated by the Particle and Pore Analysis module included in the SPIP 5.1.4 software.⁵⁴

***In situ* Atomic Force Microscopy (AFM).**

All *in situ* AFM images were captured in tapping mode at room temperature (25 °C) with a Nanoscope 8 atomic force microscope (Digital Instruments J scanner, Bruker) with hybrid probes consisting of silicon tips on silicon nitride cantilevers (HYDRA triangular lever, $k = 0.088$ N/m, tip radius < 10 nm; resonance frequency 75 kHz in air; Applied Nanostructures, Inc., www.appnano.com). The drive amplitude was about 20 nm (in fluid), and the signal-to-noise ratio was maintained above 10. The scanning rate was 1 Hz. The amplitude set point was carefully tuned to minimize the average loading force (~ 50 pN) during *in situ* imaging. The supersaturated calcium phosphate solution was made by first mixing 3.0 mM CaCl_2 with 19.0 mM Na_2HPO_4 (pH 7.40) at equal flow rates using a T junction before pumping the solution into the fluid cell at a constant rate of 3 mL/h at 25 °C, which ensured kinetically limited nucleation conditions in our systems. The supersaturation σ was 3.47 for the above solution. Supersaturation is defined as $\sigma = \ln[(\alpha\text{Ca}^{2+})^5(\alpha\text{PO}_4^{3-})^3(\alpha\text{OH}^-)/K_{\text{sp}}]$, where α denotes the species activity and K_{sp} denotes the equilibrium solubility constant at 25 °C. Speciation calculations were performed using Davies's extended Debye–Hückel equation⁵⁵ from mass balance expressions for total calcium and total phosphate with appropriate equilibrium constants by successive approximation for the ionic strength. The solubility activity products of HAP are $(3.0610 \times 10^{-7} \text{ M})$.^{9,56} Calcium and phosphate solutions were prepared immediately before use from reagent grade calcium chloride (CaCl_2 , 99.99% trace metals basis, Sigma-Aldrich) and disodium hydrogen phosphate (Na_2HPO_4 , 99.95% trace metals basis, Sigma-Aldrich) in water (pH 7.40). Each protein substrate was assembled immediately before calcium phosphate nucleation experiments and was briefly cleaned by flushing with 1 mL of deionized ($\sim 18 \text{ M}\Omega$) water to remove unbound proteins. Measurements were made over a range of enamel/rP172 ratios, and nucleation rates were determined from data collected shortly after the onset of nucleation, where the particle number density exhibited a linear dependence on the elapsed time. Both mica and HOPG were chosen to study the substrate effect on enamel and rP172 coassembly, which results in different mineral nucleation rates for different enamel/rP172 ratios. Data used in our analyses were typically collected within the first 1 h of each experiment.³⁹ For the *in situ* studies, we repeated each experiment twice. Due to the well-controlled conditions including the protein ratio, solution supersaturation, and flow rate, we found that the nucleation rates were very reproducible. We specifically repeated the experiments to make sure two observables were reproducible: (1) the number of nucleation events per specific area and (2) the time scale over which the nucleation events occurred.

***In situ* Raman Spectroscopy.**

In situ micro-Raman spectroscopy under an inverted microscope was used to investigate the separate and cooperative effects of rP172 and enamel on the kinetics of phase transformation of amorphous calcium phosphate in solution in real time. The supersaturated calcium phosphate solutions mentioned above ($\sigma = 3.47$) were made by injecting 100 μL of 3.0 mM CaCl_2 solution into 100 μL of 19.0 mM Na_2HPO_4 (pH 7.40) with 50 $\mu\text{g/mL}$ rP172 and different enamel concentrations. Therefore, the composition of final solution is 1.5 mM CaCl_2 , 9.5 mM Na_2HPO_4 , and 25 $\mu\text{g/mL}$ rP172 with different concentrations of enamel. The concentrations of enamel in the solution with enamel/rP172 ratio of 1:50, 2:50, 4:50, 8:50, 15:50 are 0.5, 1.0, 2.0, 4.0, and 7.5 $\mu\text{g/mL}$ with 25 $\mu\text{g/mL}$ rP172. Ratios

1:0, 2:0, 4:0, 8:0 and 15:0 mean the solution contained only enamel in at the same enamel concentration as of the solution designated 1:50, 2:50, 4:50, 8:50 and 15:50, respectively. These supersaturated solutions were then put into a plastic vial with a $50\ \mu\text{m} \times 50\ \mu\text{m}$ square pinhole on the bottom (Figure S5). The pinhole was used for laser penetration from the inverted optical microscope. This design excludes the use of other solid substrates between the mineral and laser, which minimizes the substrate background signal. The Raman spectra were collected from 100 to $4000\ \text{cm}^{-1}$ under backscattering geometry by a LabRAM ARAMIS confocal Raman Microscope (HORIBA scientific, Japan) operated at a resolution of $2\ \text{cm}^{-1}$ with an excitation wavelength of $532\ \text{nm}$ and laser power of $2.5\ \text{mW}$. A $100\times$ TU Plan Fluor objective with numerical aperture of 0.90 (Nikon, USA) was used to focus the solution/air interface and collect spectra for $20\ \text{s}$. For *in situ* Raman, we double checked five different solution conditions and evaluated the reproducibility of the ACP lifetime. The relative error in the lifetime ranged from 3.5% to 7.3% . These errors did not change the observation that enamel only inhibits ACP transformation and that enamel enhances ACP transformation in the presence of rP172 at enamel/rP172 ratio of $8:50$. The centers of peaks at different time points were identified by fitting with a Gaussian function. The nuclei of ACP in solution were imaged by an inverted optical microscope, and the number of nuclei in each frame was counted manually with ImageJ.

Synchrotron-Based X-ray Scattering Experiments.

For monitoring the phases of calcium phosphate at different time points, supersaturated calcium phosphate solution was made by first mixing $3.5\ \text{mM}$ CaCl_2 with $19.0\ \text{mM}$ Na_2HPO_4 ($\text{pH}\ 7.40$) in equal volumes in an Eppendorf tube and left in a static state for a period of time between 3 and $90\ \text{min}$. The suspension was then centrifuged at $11\ 800\ \text{rpm}$ for $5\ \text{min}$, after which the supernatant was carefully withdrawn and the solids on the bottom were washed with ethanol three times. The time points are calculated from the time when CaCl_2 and Na_2HPO_4 solutions were mixed until the ethanol wash. The solids were dried in a vacuum for $6\ \text{h}$ before taking the X-ray scattering measurements.

High-energy X-ray total scattering experiments were performed at beamline 11-ID-B of the Advanced Photon Source at Argonne National Laboratory, USA. Scattering data were collected with a PerkinElmer amorphous silicon detector using the rapid-acquisition pair distribution function technique.^{57,58} Measurements of the samples, empty Kapton capillary tube, and background were made at ambient temperature in a q -range of 0 – $22\ \text{\AA}^{-1}$ using an incident radiation of $\sim 60\ \text{keV}$ ($\lambda = 0.2127\ \text{\AA}$). Corrections for sample to detector distance, tilt angle of the detector with respect to the direction of the incident radiation, and polarization were performed using Fit2D software.⁵⁹ Total scattering structure functions and pair distribution functions (PDF) were obtained using the PDF-GetX2 software.⁶⁰ A crystalline standard (CeO_2) was measured and used to calculate sample–detector distance and the instrumental resolution effect on the PDF.⁶¹

Transmission Electron Microscopy (TEM).

For study of the morphology and crystallinity of calcium phosphate at different time points, supersaturated calcium phosphate solution was made as described above for X-ray scattering. Minerals were collected at different time points by dropping $50\ \mu\text{L}$ of aged

supersaturated solution onto a lacey carbon-coated 400 mesh TEM copper grid (Ted Pella, Inc.) and kept static for 1 min, after which the nonadsorbed mineral was flushed away with 100 μL of water three times. After that, the TEM copper grids were dried in a vacuum. TEM was performed with a field emission FEI Tecnai G² F20 (Hitachi Ltd.) operated at 200 kV. Images were collected by an Eagle CCD (1024 $\times$ 1024 pixels), which was included in the Tecnai User Interface (TUI).

Scanning Electron Microscopy (SEM).

SEM was performed with a Helios 600 dual-beam focused ion beam/scanning electron microscope at 5 kV. The mineral samples on HOPG substrate were taken after AFM study, flushed with water, and dried with N₂ immediately. No coating was used before the SEM imaging.

Grand Canonical Monte Carlo (GCMC) Simulation.

In the Grand Canonical ensemble, one first chooses randomly whether a trial particle insertion or deletion is attempted. If insertion is chosen, a particle is placed with uniform probability density inside the system. If deletion is chosen, then one deletes one out of N particles randomly. The trial move is then accepted or rejected according to the usual Monte Carlo algorithm. As usual, a trial move from an original state (o) to a new state (n) is accepted with different probability expressions for insertion, deletion, and displacement.^{41,42} That is, in the insertion step, a particle is placed at a random location inside the simulation box with a probability $\text{acc}(N \rightarrow N + 1) = \min\left(1, \frac{V_z}{N + 1} \exp(-\beta(U_{N+1} - U_N))\right)$; in the deletion step, a randomly selected particle species is removed with a probability $\text{acc}(N \rightarrow N - 1) = \min\left(1, \frac{N}{V_z} \exp(-\beta(U_{N-1} - U_N))\right)$; and, in the displacement step, a particle is randomly selected and displaced with a probability $\text{acc}(o \rightarrow n) = \min(1, \exp(-\beta(U_n - U_o)))$, where activity z was used to measure the particles chemical potential μ given by the relationship of $z = e^{\beta\mu}/\Lambda^3$ outside the simulation box, $1/V$ is a factor that results from placing the particle with uniform probability anywhere inside the simulation box, β is $1/k_B T$, and Λ is the de Broglie thermal wavelength.

The code was downloaded from http://www.cchem.berkeley.edu/chem195/_monte_carlo.html and modified as necessary for our calculations. The simulation box was a cube of 50×50 lattice sites. The simulation uses the above-mentioned three standard types of GCMC insertion, deletion, and displacement moves to sample the total adsorbate and monomer distributions of enamelins (green dots) in a matrix of amelogenins (white squares).^{41,42} Fifty thousand steps were used for the GCMC simulation, and frames were output every 250 steps. The total coverage of green particles and green particles without neighbors were calculated and averaged for 200 frames in the ensemble at different activities. The relationships of total coverage of green dots and coverage of isolated green dots (“monomer”) to activity z were also calculated in the presence of one kBT repulsive or attractive interactions between nearest neighbors of green dots.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic representation showing procedure for protein complexes and subsequent calcium phosphate nucleation experiments. (a) Enamelin and rP172 mixtures at different enamel/rP172 ratios followed by water washing, (b) exposure to flowing supersaturated calcium phosphate solution, and (c) induction of nucleation.

Figure 2.

In situ AFM observations of protein assembly on muscovite mica and time sequences of *in situ* AFM images showing adsorption of proteins on muscovite mica for (a) enamel/rP172 = 0:50 (0 $\mu\text{g/mL}$ enamel and 50 $\mu\text{g/mL}$ rP172) and (b) enamel/rP172 = 4:50 (4 $\mu\text{g/mL}$ enamel and 50 $\mu\text{g/mL}$ rP172). The first time point showed more separated protein particles because of the low coverage, as coverage increases at longer time, the coalescence of protein results in the flattening in topography, then the contrast of image drops. (c) Measured adsorption kinetics of proteins for different enamel/rP172 ratios where θ is the fraction of the surface covered by protein. The straight lines are linear fits of the initial stage of the adsorption process; (d) the relationship between the kinetic constant for adsorption and the range of enamel/rP172 ratios from the fit in panel (c). All scale bars are 200 nm. The time when protein solution is added into liquid cell is defined as time = 0.

Figure 3.

In situ AFM observations of calcium phosphate nucleation and growth on rP172 and enamel/rP172 films coassembled on mica for enamel/rP172 ratios of (a) 0:50 (50 μg/mL rP172 only), (b) 1:50 (1 μg/mL enamel and 50 μg/mL rP172), (c) 2:50 (2 μg/mL enamel and 50 μg/mL rP172), and (d) 4:50 (4 μg/mL enamel and 50 μg/mL rP172). Time series of *in situ* AFM images showing ACP (bright particles) nucleation and crystallization on the protein films shown in panels (a–d) in calcium phosphate solution. No free proteins are included in calcium phosphate solution. ACP particles (bright particles) dynamically nucleate and increase in numbers in the first 30 min. (e) Dependence of the number density of nuclei on time t at five different enamel/rP172 ratios obtained from the AFM in panels (a–d) plus that for a film with an enamel/rP172 ratio of 8:50. (f) Dependence of the steady-state nucleation rate J_n on time t at five different enamel/rP172 ratios derived from slopes linearly fitted by straight lines in panel (e). The mean value is averaged between two experiments, and error bars are added by using the larger error values from the experimental data and linear fitting. The scale bars for panels (a–d) are 200 nm. The time when CaCl_2 solution is added into Na_2HPO_4 solution is defined as $t = 0$.

Figure 4.

Nucleation rates and ACP lifetimes in bulk solutions containing rP172 and enamelins as monitored by *in situ* Raman spectra and X-ray scattering. (a) Number density of nuclei vs time and (b) nucleation rate derived from panel (a) by linear fitting for a range of enamelins/rP172 ratios. (c) Raman spectra of mineral at a series of time points in the absence of either rP172 or enamelins. Symmetric stretch $\nu_1(\text{PO}_4)$ peak is used as a fingerprint of different calcium phosphate phases; the broad peak of ACP centered at 954 cm^{-1} shifts to sharp peaks of OCP and HAP centered at 960 cm^{-1} . Spectra of four common calcium phosphate minerals, dicalcium phosphate dihydrate (DCPD), dicalcium phosphate anhydrous (DCPA), OCP, and HAP, are shown for reference. (d) Center of the symmetric stretch $\nu_1(\text{PO}_4)$ peak at different times for a range of enamelins/rP172 ratios (protein concentration: 0 $\mu\text{g}/\text{mL}$ enamelins and 25 $\mu\text{g}/\text{mL}$ rP172 in 0:50; 0.5 $\mu\text{g}/\text{mL}$ enamelins and 25 $\mu\text{g}/\text{mL}$ rP172 in 1:50; 1 $\mu\text{g}/\text{mL}$ enamelins and 25 $\mu\text{g}/\text{mL}$ rP172 in 2:50; 2 $\mu\text{g}/\text{mL}$ enamelins and 25 $\mu\text{g}/\text{mL}$ rP172 in 4:50; 4 $\mu\text{g}/\text{mL}$ enamelins and 25 $\mu\text{g}/\text{mL}$ rP172 in 8:50; 7.5 $\mu\text{g}/\text{mL}$ enamelins and 25 $\mu\text{g}/\text{mL}$ rP172 in 15:50). Spectra dominated by ACP and HAP are colored blue and purple, respectively. (e) Lifetime of ACP at different enamelins/rP172 ratios derived from panel (d). (f) Pair distribution functions (PDF) of mineral phase collected from solution at different times in the absence of rP172 or enamelins. The time when CaCl_2 solution is added into Na_2HPO_4 and protein mixed solution is defined as $t = 0$.

Figure 5.

Simulation of random deposition of protein particles (represented by green dots) onto a surface in the absence of calcium phosphate to determine the number of isolated monomers (out of the total number of adsorbed proteins) in a Grand Canonical Monte Carlo (GCMC) simulation. (a) Scheme of GCMC simulation in which the simulated box is an equilibrium of environment with controlled chemical potential μ . The displacement, insertion, and deletion of green dots are judged by distinct energy criteria (see main text). (b) Equilibrium snapshots of configurations of green dots for four typical activities ($z = e^{\beta\mu/\Lambda^3}$), which is a measurement of the chemical potential μ (see Experimental Procedures for details). (c) Relationship between the total coverage of adsorbed proteins (all green dots) and the coverage of isolated proteins (isolated green dots) with respect to z . (d) Relationship between coverage of monomers (isolated green dots) and the ratio by weight of occupied sites to unoccupied sites (green dots/white squares) based on the properties of enamel monomers (green dots) and rP172 oligomers (white squares) (the molecular weights of enamel monomers and rP172 monomers are 32 kDa and 19.6 kDa, respectively; the

average aggregation number of rP172 monomers inside the oligomers is 17.8 with an average height of 2.4 nm for the calculated weight ratio).⁵⁴ Both panels (c) and (d) are derived for a lattice number of 50×50 sites. Each point is the average of 200 frames in the ensemble.