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Regulation of calcium phosphate formation by native amelogenins *in vitro*

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Abstract

Our previous *in vitro* studies have shown that recombinant full-length porcine amelogenin rP172 can transiently stabilize amorphous calcium phosphate (ACP) and uniquely guide the formation of well-aligned bundles of hydroxyapatite (HA) crystals, as seen in the secretory stage of amelogenesis. This functional capacity is dependent on the hydrophilic C-terminal domain of full-length amelogenin. However, we have also found that native phosphorylated (single S-16 site) forms of full-length (P173) and C-terminal cleaved (P148) amelogenins can stabilize ACP for > 2 d and prevent HA formation. The present study was carried out to test the hypothesis that, at reduced concentrations, native full-length P173 also has the capacity to guide ordered HA formation. The effect of P148 and P173 concentrations (0.2 – 2.0 mg/ml) on the rate of spontaneous calcium phosphate precipitation was monitored via changes in solution pH, while mineral phases formed were assessed using TEM. At higher P173 concentrations (1.0 - 2.0 mg/mL), limited mineral formation occurred and only ACP nanoparticles were observed during a 48 h period. However, at 0.4 mg/mL P173, a predominance of organized bundles of linear, needle-like HA crystals was observed. At 0.2 mg/mL of P173, limited quantities of less organized HA crystals were found. Although P148 similarly stabilized ACP, it did not guide ordered HA formation, like P173. Hence, the establishment of the hierarchical enamel structure during secretory stage amelogenesis may be regulated by the partial removal of full-length amelogenin via MMP20

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Keywords

enamel matrix protein; enamel; kinetics; mineralization; amorphous calcium phosphate; hydroxyapatite

Introduction

Dental enamel, the hardest tissue in the body, is almost completely comprised (> 95% by weight) of a hierarchically ordered calcium phosphate mineral phase. Mature enamel consists of nanoscale-sized carbonated hydroxyapatite (HA) crystallites arranged anisotropically in tightly packed bundles called rods. These rods contain tens of thousands of mineral crystallites with their c-axis co-aligned with the long axis of the rods, which are further organized into distinct interwoven patterns to optimize the mechanical properties of enamel. Only trace amounts of enamel matrix proteins, secreted by ameloblasts during the initial stages of enamel formation, are found in the mature tissue due to their proteolytic processing and removal during later stages of development. The most abundant protein within this enamel matrix is amelogenin. While amelogenin is known to be essential for proper enamel formation, the mechanism by which this protein serves to guide enamel formation is still not well understood. Enamel mineralization occurs soon after enamel protein secretion (1). Full-length amelogenin is found exclusively within newly formed enamel and is believed to play a key role in the enamel organization process. This latter conclusion is supported, in part, by a number of *in vitro* studies conducted using recombinant forms of full-length amelogenin (*e.g.*, rP172 from pig), that primarily lack (2) phosphorylation of the serine-16 (S-16) site found in the native full-length amelogenin (*i.e.*, P173 from pig). Although phosphorylation has been found to affect the self-assembly full-length amelogenin in subtle but potentially important ways, it has a marked effect on crystal formation (*e.g.*, 3, 4). While oriented, well-organized bundles of crystals are observed in the presence of rP172, P173 was found to stabilize initially formed nano-particles of amorphous calcium phosphate (ACP) and prevent HA formation for up to 3 days *in vitro* (3, 4). Recently, however, we have found that P173 cleavage by MMP20 (enamelysin) can induce the transformation of stabilized ACP into ordered bundles of HA crystals, suggesting that the formation of the hierarchical enamel structure may be regulated by the proteolysis of full-length native amelogenin, during the early stages of enamel formation [unpublished findings]. During proteolysis, the onset of HA formation may be explained by two possible factors: 1) a decrease in P173 concentration; or 2) the release of functionally important cleavage products. The present study was designed to test the hypothesis that native (phosphorylated) full-length amelogenin P173 has the unique potential to regulate organized crystal formation, as seen *in vivo*, by carrying out *in vitro* mineralization studies under altered kinetic conditions that result from using varying protein concentrations. To more critically test this hypothesis, experiments were conducted using the major native proteolytic amelogenin cleavage product P148, which was similarly found to stabilize ACP *in vitro* (5).

Methods

Preparation of Porcine Amelogenins

Full-length native porcine amelogenin P173 and its predominant cleavage product P148 were isolated and purified from developing tooth buds, as previously reported (6). Absent in their recombinant counterparts, native proteins have a single phosphate group at serine-16 (S-16) and an N-terminal methionine. P148 differs from the full-length P173, by lacking the 25 amino acid hydrophilic C-terminus. Lyophilized proteins were weighed and dissolved in distilled deionized water to yield stock solutions of 5 mg/mL. Protein solutions had pH values below 3 and were maintained at 4°C for at least 24 h to ensure complete dissolution before use. Complete dissolution of protein samples was confirmed using dynamic light scattering (7). Protein stock solutions were centrifuged ($11,340 \times g$, Eppendorf Centrifuge 5403) at 4°C for 20 min, just prior to use to remove dust and any particulate matter.

Mineralization studies

Stock solutions of calcium (30 mM) and phosphate (3 mM) were prepared using reagent grade CaCl_2 (Sigma, St. Louis, MO, >99.0% pure) and KH_2PO_4 (Sigma, St. Louis, MO, >99.0% pure). All solutions (except protein solutions) were filtered (0.22- μm Isopore filters, Millipore, Billerica, MA) before use. The KH_2PO_4 solution was adjusted to pH 7.5–11.2 at 25°C, using a small volume of KOH. The precise pH value was selected by design so that the reaction solution would have an initial pH ~ 7.4 at 37°C upon mixing all solution components. Aliquots of calcium and pH-adjusted phosphate solution were sequentially added to protein solutions to yield final concentrations of 2.5 mM Ca^{2+} , 1.5 mM P_i , and 0.2–2.0 mg/mL protein, with a final volume of 60 μL , as previously reported (3, 5). Samples were then placed in a thermostatic water bath adjusted to 37°C. Initial pH values were $\sim$ pH 7.4. To minimize evaporation, the reaction tube was tightly sealed with a cap or Parafilm (America National Can, Chicago, IL). Each experiment was carried out using two identically prepared samples. In one sample, a micro-combination pH electrode (MI-410, Microelectrodes Inc., Bedford, NH) was immersed in the reaction solution to monitor changes in pH as a function of time. The other sample was used for transmission electron microscopy (TEM) analyses, as described below. Mineral phase identification was carried out using TEM in selected area electron diffraction (SAED) mode. Experiments were repeated 8–10 times.

TEM analyses of mineral products

From each tube at specified times, duplicate 5 μL aliquots were taken for TEM analyses during the mineralization reactions. Aliquots, placed on carbon-coated copper grids (Electron Microscopy Sciences, Hatfield, PA), were then processed and examined by TEM in both bright field and SAED modes, as previously described (4, 5).

Results

The effect of the varying concentrations of P173 and P148 on the rate of spontaneous calcium phosphate precipitation was monitored via changes in pH. In the absence of protein (control), as previously shown (5), the mineralization conditions used in this study induce

the initial formation of ACP (generally within the first 15 min) that subsequently transform to HA, causing a relatively rapid pH drop. This is followed by a slower pH drop (Fig. 1, curve (A)) to form randomly oriented plate-like apatitic crystals after ~1 h, as shown in Fig. 2A at 48 h. At high P173 concentrations (1.0 - 2.0 mg/mL), solution pH values remained relatively stable throughout the 48 h observation period (Fig. 1, curves (D) and (E)), consistent with the finding of very limited mineral formation (Fig. 2B). From TEM images, only nanoparticles of ACP were found during this time period, as we have previously demonstrated (3, 4). As shown (Fig. 2B), TEM and SAED (inset) analyses are consistent with the amorphous nature and known spherical morphology of ACP. However, at a significantly reduced P173 concentration of 0.4 mg/mL, a slow and gradual decrease in pH (pH 7.4 to pH 6.9) was observed over the first 32 h, followed by a more rapid decrease to pH 6.3 (Fig. 1, curve (C)). Under these conditions, TEM images showed the predominant formation of organized bundles of linear, needle-like HA crystals (Fig. 2C, inset). At an even lower P173 concentration (0.2 mg/mL), although a significant pH drop occurred at 20 h (Fig. 1, curve (B)), only limited quantities of relatively less organized HA crystals were found (Fig. 2D). Under the same mineralization conditions, P148 concentrations from 2 - 0.4 mg/mL were found to stabilize ACP for 48 h (Fig. S2), consistent with a lack of significant changes in solution pH during this time period (Fig. S1). However, at 0.4 mg/mL of P148, even though solution pH did not decrease, some randomly organized HA crystals were formed along with a clear majority of ACP nanoparticles (Fig. S2). At the lower P148 concentration of 0.2 mg/mL, however, a marked pH decrease was observed at 4 h (Fig. S1), which corresponded to the formation of randomly organized HA crystals (Fig. S2) similar to those seen in controls.

Discussion

The present results clearly show that over a wide range of concentrations both P173 and P148 dramatically inhibit spontaneous calcium phosphate formation *in vitro*, by stabilizing ACP and preventing its transformation to HA. However, at a defined concentration under a given set of conditions (*i.e.*, 0.4 mg/mL), P173 has the capacity to guide the formation of organized bundles of apatitic crystals, unlike truncated P148. Although P148 can similarly stabilize ACP nanoparticles and prevent HA formation at high concentrations like P173, P148 lacks the capacity to regulate ordered HA formation at lower concentrations. This difference may be related, in part, to our earlier findings that native (P173) and recombinant non-phosphorylated (rP172) full-length amelogenins can similarly self-assemble to form higher-order chain-like structures, while their truncated counterparts (P148 and rP147) that lack the hydrophilic C-terminus do not (4). Hence, the present findings show that under the proper kinetic conditions, regulated here by protein concentration, lower concentrations of P173 can guide the transformation of initially formed ACP nanoparticles to ordered bundles of HA crystals that have similar morphology to initially formed enamel crystals. Although the kinetics of protein-mediated mineralization processes are likely different *in vivo* from that in our experimental system, our results clearly show that P148 lacks the capacity to regulate ordered mineralization even at reduced protein concentrations, in contrast to P173. These results may suggest that, upon secretion, phosphorylated full-length amelogenin initially regulates premature mineralization of HA crystals in forming enamel. The first

mineral phase in developing enamel is ACP that later transforms into HA (8). As shown in this study, P173 can dramatically inhibit spontaneous calcium phosphate formation *in vitro* and stabilize ACP, thus preventing its transformation to apatitic material. However, full-length amelogenin undergoes proteolytic processing soon after secretion. In the developing porcine enamel, P148 is the predominate cleavage product and represents 49.5 % of total amelogenin, while P173 is reduced to only 7.4 % of amelogenin present (9). Hence, it is conceivable that the onset of enamel crystal formation may be regulated by a marked reduction (>10-fold) in the concentration of P173 to reach a critical P173 concentration that has the potential to organize forming mineral deposits into oriented bundles of elongated HA crystals, as seen in the present *in vitro* study. At very low concentration (*e.g.* < 0.2 mg/mL) of P173, the influence of hydrophilic C-terminus is lost, suggesting that the cooperative amelogenin-mediated mineralization process is concentration dependent. Although the critical protein concentration needed may be dependent on other solution factors (*e.g.*, mineral ion levels, pH, ionic strength), present results clearly show that a reduction in the concentration of full-length phosphorylated P173 can result in appropriate kinetic conditions that lead to the formation of ordered bundles of HA crystals *in vitro*. As noted above, recent studies in our laboratory have also shown that similar mineral products can be obtained through the *in situ* degradation of P173 by added MMP20. In contrast, based upon the present findings, higher concentrations of P148 found in developing porcine enamel may serve an important role by preventing uncontrolled mineralization throughout the secretory stage of amelogenesis where the volume of mineral deposits is only 15-20% (10).

Conclusions

The generation of the hierarchical enamel structure during secretory stage amelogenesis may be regulated by the partial removal of full-length amelogenin via proteolysis, while predominant amelogenin degradation products, like P148, serve to prevent uncontrolled mineral formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Changes in pH observed during mineralization experiments as a function of time and P173 concentration: (A) in the absence of protein, (B) 0.2 mg/mL, (C) 0.4 mg/mL, (D) 1.0 mg/mL, and (E) 2.0 mg/mL. Similar results were obtained for multiple repeats ($n = 8 - 10$).

Figure 2. Transmission electron microscopy (TEM) and selected area electron diffraction (SAED) (insets) analyses of mineral phases formed in the presence of various concentrations of P173; (A) in the absence of protein (control), (B) 1.0 mg/mL, (C) 0.4 mg/mL, and (D) 0.2 mg/mL. The broad and diffuse SAED ring pattern in (B) is consistent with that of amorphous calcium phosphate (ACP), while the presence of discrete diffraction spots and sharp rings seen in (A) and (D) are consistent with randomly arranged hydroxyapatite (HA) crystals. Narrow diffraction arcs at (002) lattice position in (C) indicate the presence of bundles of well-oriented HA crystals.