Role of 20-kDa Amelogenin (P148) Phosphorylation in Calcium Phosphate Formation in Vitro

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The potential role of amelogenin phosphorylation in enamel formation is elucidated through in vitro mineralization studies. Studies focused on the native 20-kDa porcine amelogenin proteolytic cleavage product P148 that is prominent in developing enamel. Experimental conditions supported spontaneous calcium phosphate precipitation with the initial formation of amorphous calcium phosphate (ACP). In the absence of protein, ACP was found to undergo relatively rapid transformation to randomly oriented plate-like apatitic crystals. In the presence of non-phosphorylated recombinant full-length amelogenin, rP172, a longer induction period was observed during which relatively small ACP nanoparticles were transiently stabilized. In the presence of rP172, these nanoparticles were found to align to form linear needle-like particles that subsequently transformed and organized into parallel arrays of apatitic needle-like crystals. In sharp contrast to these findings, P148, with a single phosphate group on serine 16, was found to inhibit calcium phosphate precipitation and stabilize ACP formation for more than 1 day. Additional studies using non-phosphorylated recombinant (rP147) and partially dephosphorylated forms of P148 (dephos-P148) showed that the single phosphate group in P148 was responsible for the profound effect on mineral formation in vitro. The present study has provided, for the first time, evidence suggesting that the native proteolytic cleavage product P148 may have an important functional role in regulating mineralization during enamel formation by preventing unwanted mineral formation within the enamel matrix during the secretory stage of amelogenesis. Results obtained have also provided new insights into the functional role of the highly conserved hydrophilic C terminus found in full-length amelogenin.

Extracellular matrix molecules play a crucial role in the regulation of biological mineralization by controlling crystal size, shape, and organization. An example of this exquisite regulation is in the formation of the highly organized dental enamel tissue that is regulated in part by amelogenin, the major extracellular matrix protein secreted by ameloblasts (1). Although amelogenin is processed by proteinases soon after secretion, the intact full-length parent molecule has been found to be exclusively associated with newly formed enamel mineral (2). Prior studies in our laboratory (3) have also shown that full-length recombinant mouse amelogenin (rM179) can regulate the formation of parallel arrays of apatitic crystals (a salient feature of developing and mature dental enamel) under conditions of spontaneous precipitation in vitro. This functional capability appears to be related to the specific primary structure of the full-length amelogenin and its unique assembly properties under certain physicochemical conditions of pH and temperature (4). In particular, the conserved hydrophilic C terminus of amelogenin has been shown to play a key role in these processes (3).

To date, however, most studies have utilized recombinant amelogenins that lack the N-terminal methionine and the single phosphate group (Ser-16) found in native proteins (e.g. Fig. 1). Thus, the effect of phosphorylation on amelogenin self-assembly and its regulation of mineralization is not known. This report describes for the first time evidence that the single phosphate group found in native amelogenins has a profound influence on the formation of calcium phosphates in vitro. The potential role of amelogenin phosphorylation in enamel formation is elucidated here through comparative studies using the 20-kDa porcine amelogenin proteolytic cleavage product P148, found in the developing enamel matrix, and its recombinant form (rP147) that lacks the N-terminal methionine and the single phosphate group (Ser-16). P148 has been shown to be the predominant cleavage product present during the secretory stage of developing porcine enamel (5, 6). The recombinant full-length amelogenin (rP172) was also investigated for further comparison, providing additional new insights into the role of the hydrophilic C terminus. The smaller molecules used (rP147 and P148) lack the hydrophilic C terminus present in the full-length parent molecule (Fig. 1).

EXPERIMENTAL PROCEDURES

Preparation of Porcine Amelogenins—The predominant native porcine amelogenin cleavage product P148 was isolated and purified from developing tooth buds as previously described (6). The recombinant analog of P148, rP147, and the recombinant full-length amelogenin from pig, rP172, were pro-
The precise pH value was selected by design so that the reaction solution (including protein) would have an initial pH value of ~7.4 at 37 °C upon mixing of all solution components. With the exception of the centrifuged protein solution, all solutions were filtered (0.22-μm Isopore filters (Millipore)) prior to use.

Mineralization Studies—Aliquots of calcium and the pH-adjusted phosphate solution were sequentially added to cold protein solutions maintained on ice to yield final concentrations of 2.5 mM Ca$^{2+}$, 1.5 mM Pi, and 2 mg/ml protein, with a final volume of 60 μl. The samples were then placed in a thermostatic water bath adjusted to 37 °C. Initial pH values were ~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 microcombination 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)3 analyses, as described below. Additional experiments were also carried out over a 4-h period using a larger sample volume (1.8 ml) that was stirred with a small magnetic stir bar. At the end of this time period, in selected experiments changes in total calcium and phosphate were determined using atomic absorption spectroscopy (AAAnalyst 200, PerkinElmer Life Sciences) and a spectrometric method (8), respectively. Mineral phase identification was carried out using TEM in selected area electron diffractogram (SAED) mode and Fourier transform-infrared (FT-IR) spectroscopy.

TEM Analyses of Mineral Products—5-μl aliquots were taken for TEM analyses at specified times during the mineralization reactions. These aliquots were placed on carbon-coated copper grids (Electron Microscopy Sciences, Hatfield, PA) for 0.5–1 min, blotted vertically against filter paper, quickly rinsed with distilled water, blotted again, and then air-dried. Grids were prepared in duplicate. Images were obtained in bright field and SAED modes using a JEOL 1200 TEM operated at 100 kV and captured by an AMT CCD camera (AMT, Danvers, MA). The analysis of micrographs was performed using NIH ImageJ 1.64 software.

FT-IR Spectroscopy Analyses—Following each mineralization experiment, reaction mixtures were concentrated by centrifugation and the removal of 20 μl of the supernatant. A 20-μl
aliquot of the concentrated sample was then placed on a KBr sheet (KBr IR Card, International Crystal Labs, Garfield, NJ) and dried overnight in a vacuum desiccator or at room temperature. However, to prevent possible phase transformation of ACP, spectroscopic grade acetone was added to concentrated 1-day samples prepared in the presence of P148 before overnight drying. FT-IR spectra (4000 to 450 cm$^{-1}$) of these samples were then recorded using a PerkinElmer Multiscope FT-IR microscope. FT-IR results are presented in the supplemental material.

RESULTS

Mineralization Kinetics in the Presence and Absence of Porcine Amelogenins—The effect of rP147, P148, and rP172 on the rate of spontaneous calcium phosphate precipitation was monitored via changes in pH. In the absence of protein (control), using 60-μl samples, a decrease in pH was observed to take place in three distinct steps, corresponding to an initial induction period of ~15 min associated with the formation of amorphous calcium phosphate (ACP), as shown below, followed by a rapid and a slower apatitic crystal growth phase (Fig. 2A, curve a). A similar change in pH with time was also observed in the presence of the full-length recombinant amelogenin rP172 (Fig. 2A, curve b), following a longer ~45-min induction period, although the pH was found to level off (at ~6 h) at a higher value than that seen in the control. In the presence of recombinant amelogenin rP147 that lacks 25 C-terminal amino acids, however, an almost immediate decrease in pH was observed (Fig. 2A, curve c). Although the initial pH drop is similar to that seen in the control, the pH leveled off (after 4 h) again at a higher value than observed in the control. In sharp contrast to these findings, however, the native phosphorylated form of rP147, P148 (Fig. 1), behaved quite differently under the same experimental conditions. In 60-μl samples, in the presence of P148, relatively little pH change was observed for 1 day (Fig. 2A, curve e) and even longer (i.e. up to 3 days (data not shown)). At the beginning of the reaction, the pH decreased slightly (0.2–0.4 pH units) during the first hour and then slowly increased to almost the initial pH value that remained relatively constant for up to 24 h, as shown. Marked changes in pH as seen in the control, or in the presence of rP172 and rP147, were not observed. Similar findings were also obtained when a larger (stirred) reaction volume (1.8 ml) was used. A mineralization induction time (~45 min) was observed for rP172 that was again greater than that seen in the control (~20 min), while pH values remained nearly constant for the 4-h period in the presence of P148 (Fig. 2B, curve c) following a small initial decrease. Relative changes in pH at 4 h were also reflected in observed changes in total calcium and phosphate concentrations measured in selected samples (Table 1) that showed, in general, a much smaller decrease in mineral ion concentrations in the presence of P148 in comparison to those observed with rP172 and in the control. These changes in mineral ion concentrations are also consistent with the relative amounts of mineral observed in 60-μl samples using TEM, as described below. When larger volumes of stirred solutions were monitored for pH for longer times, pH values did not change for up to 10 h in the presence of P148 (data not shown).

**TABLE 1**

| Samples | Calcium (%) | Phosphate (%) | n |
|---------|-------------|---------------|---|
| Control | 47.5 (7.3)  | 47.4 (8.0)    | 6 |
| rP172   | 56.1 (4.7)  | 58.6 (3.7)    | 4 |
| P148    | 19.4 (6.5)  | 12.1 (3.8)    | 2 |

**FIGURE 2.** A, changes in pH as a function of time observed during mineralization experiments carried out in 60-μl sample volumes, in the absence (curve a) and presence of amelogenin: rP147 (curve b), rP172 (curve c), dephos-P148 (curve d), and P148 (curve e). B, changes in pH as a function of time observed during mineralization experiments carried out in 1.8-ml sample volumes (with stirring) in the absence (curve a) and presence of amelogenin rP172 (curve b) and P148 (curve c).
were consistent with the formation of hydroxylapatite (HA), as confirmed by FT-IR (see supplemental material).

TEM and SAED analyses of sample solutions containing rP172 also revealed the initial (~15 min) presence of nanometer-sized ACP particles, as seen in Fig. 3B (and inset). In general, these particles were smaller than those seen in the control, having diameters of 8.5 ± 1.1 nm (n = 166). At 45 min (Fig. 3B), small nanoparticles were seen to align and form linear needle-
like particles, whereas after 1–4 h needle-like crystals were observed. After 1 day, multiple bundles of aligned needle-like crystals (l = 154 ± 35 nm; n = 91) were seen. The SAED pattern of an isolated bundle exhibited narrow arcs corresponding to 002 and 004 reflections, as shown in Fig. 3B (1 day, insets), indicating that the thin crystals were elongated in the [001] direction and that the crystals were organized into bundles with their crystallographic c-axes preferentially oriented in near parallel arrangement. SAED results were again consistent with the formation of HA, as confirmed by FT-IR (see supplemental material).

When rP147, a recombinant truncated version of rP172, was used, randomly distributed plate-like HA crystals, based on SAED and FT-IR analyses (see supplemental material), were observed at 1 day that were similar in these respects to those seen in the control (Fig. 3D, 1 day and inset), but unlike those observed in the presence of rP172. These crystals, however, were found to be significantly (p < 0.001) though slightly smaller (l = 67.1 ± 18.5 nm, n = 79) than those in control samples. As shown in Fig. 3D, mineralization in the presence of rP147 appeared to take place outside of the observed protein masses. At 15 min, as suggested by the observance of a somewhat diffuse SAED diffraction pattern (Fig. 3D, inset), TEM analyses revealed both spherical particles and small plate-like crystals. At 45 min, diffraction spots forming a discrete ring pattern were clearly visible, as was the presence of plate-like crystals after 1–4 h of reaction.

In sharp contrast to the control and samples with rP147 and rP172, monodispersed particles and/or networks of ACP were observed by TEM in samples containing P148, the native phosphorylated derivative of rP147, at all noted time points from 15 min to 1 day (Fig. 3C). SAED patterns at 15 min and at 1 day were characteristic of ACP (Fig. 3C, insets), consistent with the lack of pH change observed during the mineralization experiments in the presence of P148. The amorphous nature of the reaction product formed (at 1 day) in the presence of P148 was confirmed by FT-IR spectroscopy (see supplemental material). Such mineral particles were rarely found, however, when the entire grid was examined (data not shown). Nevertheless, in the presence of P148, monodispersed spherical particles (d = 36.4 ± 3.6 nm, n = 60) of ACP were clearly seen by TEM at 1 day (Fig. 3C) that were similar in size and appearance to those observed in the control within the first 15 min of reaction. P148 was also found to stabilize ACP formation at a lower protein concentration of 1 mg/ml, whereas at protein concentrations of 400 μg/ml and below, apatitic crystal growth was observed (data not shown).

**Partial Dephosphorylation of P148 and Its Effect on Calcium Phosphate Formation**—Reacting native P148 with acid phosphatase reduced the percentage of Ser-16-phosphorylated sites by almost 50% from an initial value of 66.1 to 34.5%. Using the partially dephosphorylated protein (dephos-P148) in the 60-μl mineralizing system, a decrease in solution pH was observed after a 4-h induction period (Fig. 2A, curve d), in contrast to that seen for P148. The observed change in pH corresponded to the formation of bundles of plate-like crystals (Fig. 4C) that were significantly (p < 0.001) larger (l = 213 ± 55 nm, n = 98) than those seen in the control or in the presence of rP147 and rP172. To illustrate the effect of the single phosphate group present in P148, TEM results obtained at 1 day for rP147, P148, and dephos-P148 are compared in Fig. 4. A summary of the shape, size, and identification of mineral phases formed after 1 day in the absence and presence of all amelogenins studied is also presented in Table 2.

**DISCUSSION**

P148 is the predominant cleavage product of the most abundant extracellular matrix protein in the secretory stage of de-

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TABLE 2

| Amelogenins | Particle size (length, nm) | Shape | Phase |
|--------------|---------------------------|-------|-------|
| None (control) | 72.9 ± 20.6 (n = 76) | Randomly arranged plates | HA |
| rP172 | 154 ± 35 (n = 91) | Bundles of aligned needles | HA |
| rP147 | 67.1 ± 18.5 (n = 79) | Randomly arranged plates | HA |
| Dephos-P148 | 213 ± 55 (n = 98) | Bundles of plates | HA |
| P148 | 36.4 ± 3.6 (n = 60) | Spherical particles | ACP |

**FIGURE 4.** TEM micrographs of calcium phosphate mineral products formed in the presence of amelogenins: rP147 (A), P148 (B), and dephos-P148 (C) examined after 1 day. This is described in more detail under “Experimental Procedures.” As discussed in the text, plate-like apatitic crystals that were slightly but significantly smaller than those found in the control (Table 2) were observed when mineralization was carried out in the presence of the non-phosphorylated rP147 (A), whereas bundles of significantly larger plate-like crystals were observed when mineralization was carried out using the partially dephosphorylated P148, dephos-P148 (C). In sharp contrast, assemblies of spherical ACP particles were observed in the presence of P148 (B).
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In the present study, the identification of ACP was partially based on observed particle morphology and diffuse electron diffraction patterns. It should be noted, however, that such amorphous scattering patterns might also result from the presence of agglomerations of nanocrystals of apatitic material (11). Although some uncertainty remains, in the absence of high-resolution TEM analyses of the observed spherical particles, our SAED findings agreed well with FT-IR analyses that were clearly consistent with the presence of ACP (see supplemental material).

Collectively, our results show that phosphorylation has a profound effect on the ability of P148 to inhibit apatite formation under conditions that support the spontaneous formation of calcium phosphates. In vivo, therefore, P148 may play an important functional role in regulating mineralization during enamel formation through its inhibition of crystal nucleation and the stabilization of ACP. This inhibition may serve to prevent unwanted mineral formation throughout the majority of the extracellular enamel matrix during the secretory stage of amelogenesis where very thin crystals of enamel mineral are formed and constitute only ~10–20% of the extracellular matrix volume, with the remaining portion occupied by protein and water (12, 13). Consistent with our observations regarding P148 and in support of our experimental model that uses highly supersaturated calcium phosphate solutions that induce ACP formation, data have been obtained that suggest the presence of ACP in the developing enamel matrix (14–16). Transient amorphous mineral phases (i.e. ACP and amorphous calcium carbonate) have also been observed to be associated with a number of other biomineralization systems, including mollusk shells and teeth, echinoderm skeletal elements, sponge spicules (17–21), and bone (10, 22, 23). Other molecules believed to be associated with the regulation of biomineralization, like the highly phosphorylated osteopontin from milk (24) and the N-terminal domain of dentin matrix protein 1 (25), have been shown to stabilize amorphous calcium phosphate. Furthermore, it has been proposed that the transformation of amorphous phases to crystalline materials is an important aspect of the biological mineralization process (21, 26, 27). Our data show that, under the chosen experimental conditions, relatively large ACP particles initially form in the absence of protein and subsequently transform to randomly arranged apatitic crystal plates (Fig. 3A). Unlike P148, rP172 was found to only transiently stabilize ACP and its transformation to apatitic crystals.

In comparison to these findings obtained under conditions of spontaneous precipitation of calcium phosphates, it has been reported (28) that the 25-kDa full-length porcine amelogenin (i.e. P173) selectively adsorbs onto synthetic HA crystals and partially inhibits their growth when added to a metastable solution supersaturated with respect to HA. However, the 20-kDa P148 degradation product was found to adsorb substantially less onto HA crystals and did not significantly inhibit HA crystal growth. The lower affinity of P148 for HA crystals, compared with P173 and rP172, was subsequently demonstrated in another study (29). In light of these reported results, our current findings suggest that P148 interacts more strongly with critical nuclei precursors and ACP than with mature HA crystals.

Although the mechanism by which molecules stabilize amorphous phases is not well understood, structure-function studies (30–32) have demonstrated that the propensity of a molecule to inhibit spontaneous (primary) precipitation of HA, as compared with seeded (secondary) HA crystal growth, can be related to specific structural domains within the molecule. In particular, it was shown (30) that the ability of the salivary protein statherin to inhibit primary precipitation was mostly related to its hydrophobic C terminus, whereas its ability to inhibit secondary precipitation was related to the N-terminal hexapeptide containing two phosphoserines. The authors of this study proposed that, with respect to inhibition of primary precipitation, the highly charged N-terminal region interacts with the initially formed calcium phosphate nuclei and that the hydrophobic C terminus (presumably) oriented toward the aqueous solution would restrict the diffusion of mineral ions toward the forming crystal embryo (30). Based on our present findings, a similar mechanism for the inhibition of calcium phosphate formation by P148 could be suggested, where the phosphorylated N-terminal region binds strongly to forming calcium phosphate nuclei and ACP, and their transformation to HA is inhibited, in part, due to the presence of a rather large predominantly hydrophobic C-terminal portion of the P148 molecule. Hence, one might predict that, as in the case of statherin, the subsequent removal of hydrophobic residues from the C terminus of amelogenin, as occurs in vivo, would reduce the observed inhibitory activity of native amelogenins. However, from the results obtained in the presence of the non-phosphorylated analog of P148, rP147, showing the formation of randomly oriented plate-like crystals similar to those found in the control, it is apparent that the affinity of rP147 for both ACP and forming apatitic crystals is relatively low. Based on these findings and those obtained showing that mineralization took place when the level of P148 phosphorylation (i.e. dephospho-P148), was reduced significantly, it is concluded that
the inhibitory activity of P148 is primarily due to the single phosphate group on Ser-16 that results in a high affinity for forming crystal embryos and ACP.

Despite the lack of the Ser-16 phosphate group, rP172, which contains the 25-amino acid hydrophilic C terminus, was found in the present study to interact with ACP transiently, stabilizing this mineral phase for relatively brief periods of time (<1 h) in comparison to P148, as well as with forming apatitic crystals as ACP transforms, affecting crystal shape and organization. In comparison to results obtained using rP147, showing no specific effect on mineral formation, these latter results indicate that the presence of the hydrophilic C terminus enhances amelogenin interactions with mineral phases, consistent with earlier findings (3, 28, 29, 33). Observed differences between rP147 and rP172 interactions with mineral phases, however, may also reflect differences in their self-assembly properties in addition to their differences in primary structure. In a recent study (4), we found that rP172 can form tightly connected elongated assemblies of small spheres with a high aspect ratio, whereas P148 formed loosely associated networks of larger spherical particles under similar conditions. This difference in protein assembly that was attributed to the absence of hydrophilic C terminus of P148 (4) may also significantly affect protein-mineral interactions. Importantly, rP147 has been found to self-assemble in a manner similar to that observed for P148 (data not shown) and, consequently, also differs from rP172 in this respect.

Unlike the results obtained using rP147, the full-length amelogenin rP172 was found to promote the formation of ordered arrays of needle-like apatitic crystals. This result is consistent with those obtained in our previous study (3) using the full-length recombinant amelogenin (rM179) from mouse. This similarity is not surprising because the primary structure of rM179 is highly homologous with that of rP172, with nearly identical N- and C-terminal domains (1). In addition, we found previously that both rM179 and rP172 self-assemble in a similar pH-dependent fashion to form either nanometer-sized particles (nanospheres) or chain-like structures composed of closely linked nanoparticles (4). Hence, the present findings also demonstrate that the C-terminal domain that is present in rP172, and not in rP147, plays an important role in regulating the shape and organization of forming mineral crystals, under conditions where mineralization and protein assembly are induced simultaneously (3). Of considerable interest, we have obtained TEM evidence (Fig. 3B, 45 min) showing that small nanoparticles (presumably ACP) align and form linear needle-like structures in the presence of rP172 (Fig. 3B), during the transformation of ACP particles to aligned bundles of apatitic crystals (1 day). This finding is consistent with a proposed hypothesis that initial enamel crystals form from the assembly and fusion of amorphous calcium phosphate particles (e.g. Ref. 34), as supported by very recent findings (16). It has also been recently suggested that such assembly and fusion processes are facilitated by the presence of ACP (35). Furthermore, it has been reported (36) that chain-like structures composed of amorphous nanoparticles, suggestive of the formation of ACP, were observed in solution in the presence of rP172 prior to the electrolytic deposition of crystalline calcium phosphate on a cathode surface. The identification of ACP in this study, however, was based solely on the lack of evidence of crystalline material using SAED. Experimental conditions used in this noted study also differed significantly from those used in the present study. In vitro studies are currently under way to explore the effect of the phosphorylation of Ser-16 on the self-assembly and function of the full-length porcine amelogenin.

Finally, it should be noted that the present in vitro studies were carried out using amelogenin concentrations (0.2–2.0 mg/ml) that are below those found (12, 38) in secretory enamel (200–300 mg/ml) and at an ionic strength lower than that found in enamel fluid (39). Such factors must ultimately be considered with respect to the biological relevance of in vitro findings. Nevertheless, the protein structure-function relationships described here have provided important new insights into amelogenesis processes, under experimental conditions that yield mineral phases and patterns of crystal organization similar to those in developing enamel.

In contrast to the results presented here using native and recombinant proteins, it has recently been reported (40–42) that very low concentrations (0.5–6.5 μg/ml) of rP172 accelerate HA nucleation kinetics in a concentration-dependent manner. These studies were carried out in modestly supersaturated solutions that undergo spontaneous formation of HA in ~700 h under near-physiological pH, temperature, and ionic strength conditions. However, at somewhat higher concentrations (65 μg/ml) the induction time was found to increase (relative to that observed at 6.5 μg/ml) and, lesser amounts of HA precipitated (41). It has also been reported (43) that higher concentrations (1.6 mg/ml) of recombinant full-length human amelogenin (rH174) accelerated the growth of a mineral layer (based on AFM height measurements) on fluoroapatite crystals, relative to controls, under driving forces for mineralization that were lower than those used in the present study. Again, an acceleration of nucleation and crystal growth was not observed in the present study that was carried out at higher protein concentrations and under higher driving forces for precipitation where HA formation proceeded through the formation of ACP. The latter conditions used in the present study, however, may better reflect the in vivo situation, for the reasons already noted. Nevertheless, it is apparent that protein-mineral interactions with respect to the promotion and inhibition of crystal formation are highly dependent on environmental conditions (37).

In conclusion, we have presented for the first time evidence that the single phosphate group found in native amelogenins can have a profound influence on the formation of calcium phosphates in vitro. Evidence obtained suggests that native propeolytic cleavage products like P148 may have a functional role during enamel formation. Moreover, we have provided new insights into the mechanism of crystal formation and the functional role of the highly conserved hydrophilic C terminus.

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