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CryoTEM Study of Effects of Phosphorylation on The Hierarchical Assembly of Porcine Amelogenin and Its Regulation of Mineralization *in vitro*

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Abstract

Amelogenin, the major extracellular enamel matrix protein, plays a critical role in regulating the growth and organization of enamel. Assembly and mineralization of full-length native (P173) and recombinant (rP172) porcine amelogenins were studied by cryogenic Transmission Electron Microscopy (cryoTEM). The cryoTEM revealed that both native and recombinant porcine amelogenins undergo step-wise self-assembly. Although the overall structural organization of P173 and rP172 oligomers was similar and resembled oligomers of murine recombinant amelogenin rM179, there were subtle differences suggesting that a single phosphorylated serine present in P173 might affect amelogenin self-assembly. Our mineralization studies demonstrated that both P173 and rP172 oligomers stabilize initial mineral clusters. Importantly, however, rP172 regulated the organization of initial mineral clusters into linear chains and guided the formation of parallel arrays of elongated mineral particles, which are the hallmark of enamel structural organization. These results are similar to those obtained previously using full-length recombinant murine amelogenin (Fang et al., 2011a). In contrast to that seen with rP172, phosphorylated P173 strongly inhibits mineralization for extended periods of time. We propose that these differences might be due to the differences in the structural organization and charge distribution between P173 and rP172. Overall our studies indicate that self-assembly of amelogenin and the mechanisms of its control over mineralization might be universal across different mammalian species. Our data also provide new insight into the effect of phosphorylation on amelogenin self-assembly and its regulation of mineralization.

Keywords

BIOMINERALIZATION; CRYO TRANSMISSION ELECTRON MICROSCOPY; SINGLE PARTICLE RECONSTRUCTION; SELF-ASSEMBLY; ENAMEL; AMELOGENIN

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Introduction

Dental enamel is a unique and structurally complex nanocomposite material comprising the outer layer of a tooth crown. Its intricate three-dimensional organization arises from arrays of elongated apatitic crystallites known as enamel rods and identified as basic building blocks of enamel structure (Warshawsky and Nanci, 1982). The structural organization of enamel is the key for its unique mechanical properties, that combine exceptional hardness and toughness (Bajaj and Arola, 2009; Baldassarri et al., 2008; Chai et al., 2009; Zaslansky et al., 2006). This organization is tightly controlled by supramolecular assemblies of extracellular matrix molecules (Fincham et al., 1999; Margolis et al., 2006). Studies of enamel from wild type and knockout animals clearly demonstrate that extracellular matrix proteins tightly regulate mineral phase and structural organization of mineral particles in forming enamel via specific protein-mineral interactions (Beniash et al., 2009; Caterina et al., 2002; Gibson et al., 2001; Jodaikin et al., 1988; Paine et al., 2000; Pugach et al., 2010; Simmer et al., 2012).

Amelogenin, the major structural protein of dental enamel, has been shown to play a critical role in the regulation of biomineralization in forming enamel (Gibson et al., 2001; Hu et al., 2007; Paine et al., 2000). It is a modular protein which self-assembles at physiological pH into nanospheres 15–30 nm in diameter (Fang et al., 2011a; Fang et al., 2011b; Fincham et al., 1994; Fincham et al., 1995; Moradian-Oldak et al., 1998; Wiedemann-Bidlack et al., 2007). Amelogenin is comprised of three domains: an N-terminal tyrosine-rich amelogenin peptide (TRAP), a charged C-terminal hydrophilic telopeptide (C telopeptide), and a central domain rich in X-Y-Pro repeats (Margolis et al., 2006). At low pH amelogenins are globally disordered, whereas upon an increase in pH an increase in beta sheet has been reported (Beniash et al., 2012; Delak et al., 2009; Lakshminarayanan et al., 2009). *In vitro* mineralization experiments with native and recombinant protein have revealed that amelogenin can control the mineral phase, shape and organization of mineral particles (Beniash et al., 2005; Deshpande et al., 2010; Kwak et al., 2009; Kwak et al., 2011). Native amelogenin has a single phosphorylation site and it has been shown that phosphorylated native porcine amelogenin (P173) inhibits calcium phosphate crystallization and stabilizes amorphous calcium phosphate unlike its recombinant non-phosphorylated counterpart rP172 (Kwak et al., 2011; Wiedemann-Bidlack et al., 2011). In contrast to these marked differences in their effects on mineralization, our recent studies, using dynamic light scattering and room temperature TEM, demonstrate that phosphorylation of the full-length P173 has only a small, albeit potentially important, effect on its higher-order self-assembly under physiological pH conditions (Wiedemann-Bidlack et al., 2011). Furthermore, our recent cryo-TEM study of nonphosphorylated recombinant mouse amelogenin (rM179) shows that it undergoes step-wise self-assembly and that rM179 oligomers stabilize mineral pre-nucleation clusters and guide their arrangement into linear chains that organize as parallel arrays, prior to crystallization (Fang et al., 2011a).

To test the hypothesis that step-wise assembly is a universal trait of amelogenins, and to study the effects of phosphorylation on self-assembly of this protein and on its regulation of biomineralization, we have conducted a series of studies with native phosphorylated (P173) and recombinant nonphosphorylated (rP172) porcine amelogenins by cryo-TEM where protein assembly and mineralization processes to be studied in their native hydrated state. Hence, this approach can provide further insights into biological mineralization processes regulated by self-assembled macromolecules.

Materials and Methods

Preparation of Porcine Amelogenin

The full-length native porcine amelogenin P173 was isolated and purified from developing tooth buds as previously described (Yamakoshi et al., 1994). The purity of the protein preparation was 90–95%. The degree of phosphorylation of native P173, purified in this fashion, was previously found by mass spectrometry to be ~85% (unpublished).

Recombinant amelogenin rP172, was produced in bacteria and purified, as described elsewhere (Ryu et al., 1999; Simmer et al., 1994). The recombinant proteins lack a single phosphate group at serine 16 (S-16) and an N-terminal methionine. The same batches of P173 and rP172 were used for both assembly and mineralization studies.

Self-Assembly Experiments

The self-assembly experiments were carried out in a manner identical to our earlier experiments with murine amelogenin rM179 and described elsewhere (Fang et al., 2011a; Fang et al., 2011b). Protein stock solutions of 2 mg/ml were prepared by dissolution of the lyophilized proteins in ice-cold, distilled and deionized water. The solution was adjusted to pH 3.5 using HCl and kept for at least 24 hours at 4°C prior to the experiments to ensure complete dissolution of the protein.

Aliquots of stock solution were added to 4mM PBS with pH adjusted to pH 8.0, on ice to establish a final protein concentration of 100 µg/ml. Lacey carbon 400 mesh TEM grids (EMS, Hartfield, PA) were placed on top of small droplets (20 to 50 µl) of the protein solutions and incubated for different time intervals from 1 to 30 min at room temperature. The grids were then blotted and immediately plunge-frozen in ethane slush cooled by liquid nitrogen using a Vitrobot automated plunge freezer (FEI, Hillsboro, OR). The grids were transferred onto a Gatan 626 cryoholder (Gatan, Pleasanton, CA) and inserted into an FEI Tecnai F20 cryoTEM equipped with field-emission gun operating at 200 KV. Images were recorded using a Gatan 4k × 4k charge-coupled device (CCD) UltraScan 4000 camera. The images were taken under low dose mode at 20 e/Å² to minimize radiation damage to the samples. To enhance the image contrast, under-focusing in the range of 1–3 µm was used to record the images. The calibrated magnification was 70,093, resulting in a pixel size of 2.14Å.

Single Particle Reconstruction

Three dimensional single particle reconstructions of amelogenin monomers and oligomers were performed using a single-particle image processing software package EMAN (Ludtke et al., 1999). 575 and 430 particles were selected for P173 monomer and oligomer reconstruction, respectively (Supplementary Figure 1A). 558 and 512 particles were selected for rP172 monomer and oligomer reconstructions respectively (Supplementary Figure 1B). The initial models were built using 100 randomly selected raw particles. The initial orientation of individual particles was randomly assigned within the corresponding asymmetry unit. A projection-matching algorithm was then used to determine the center and orientation of raw particles in the iterative refinement until convergence. For the P173 monomer, no symmetry was imposed during the reconstruction. D6 symmetry (6 fold dihedral rotational symmetry) was imposed in the P173 oligomer and control reconstruction, along with C6 symmetry (6 fold rotational symmetry) and without imposing any symmetry to check to see that the particles indeed have these noted symmetries. Final maps were visualized using UCSF Chimera software (Goddard et al., 2005). Contour levels were chosen for the surface views to enclose 100% of the protein volume calculated from the relative molecular weights of P173 and rP172, and using an average protein partial specific volume of 0.73 cm³/g (Harpaz et al., 1994).

Mineralization Experiments

The self-assembly experiments were carried out in a manner identical to our earlier experiments with murine amelogenin rM179 and described elsewhere (Fang et al., 2011a). Stock solutions of amelogenins P173 and rP172 were mixed with CaCl_2 with Na_2HPO_4 solution to establish the final protein concentration 0.2 mg/ml, 1mM phosphate and 1.67 mM Ca^{2+} . Solution pH was adjusted to pH=8.0 by adding NaOH. The droplets of the solutions were mounted on lacey carbon grids and incubated at 37°C in a humidity chamber. The samples were taken from the humidity chambers at 10, 30, 60 and 120 minutes after the beginning of the reaction, blotted and immediately plunge-frozen into ethane slush cooled by liquid nitrogen using Vitrobot.

Results

We observed several different particle classes in the self-assembly experiments with P173 1 minute after the beginning of the reaction (Figure 1a). Representative particles of different classes are shown in Figure 1c–h. The first class contained ellipsoid particles, up to 3 nm in the longest dimension (Figure 1c). There were a number of particles of different diameters that we interpreted as being oligomers containing different numbers of protein molecules (Figure 1d, e, f). Interestingly, all oligomer classes had a low-density core surrounded by an electron dense periphery, suggesting that these oligomers adopt a cage or barrel-like structure. Another interesting particle class consists of pairs of oligomers and sometimes groups of three or more oligomers (Figure 1g). It is likely that these small groups of oligomers were caught in the process of assembly into higher order structures. After 10 minutes of incubation (Figure 1b), the majority of the particles were spherical aggregates ~20 nm and with internal structure (Figure 1h) similar to the nanospheres observed by other methods. Self-assembly of recombinant porcine amelogenin rP172, which lacks the N-terminal methionine and is not phosphorylated, closely resembled P173. Fig. 2a shows a typical cryo-TEM image of self-assembly after 1 minute of reaction at pH 8.0. In these samples, the majority of particles were monomers (Figure 2c) and oligomers of different sizes comprising different numbers of individual protein molecules (Figure 2d–f). Furthermore, pairs and chains of 3–4 oligomers were also observed. Nanospheres became the predominant particle class after 10 min incubation at pH 8.0 (Figure 2b, h).

In order to further explore the mechanism of amelogenin self-assembly process, a three-dimensional reconstruction of amelogenin P173 was calculated from 575 manually selected monomers. Figure 3a shows three views of the reconstructed monomers, one top, and two side views at 90° angle to each other. The monomers have an ellipsoid shape, a foot-like process at one of its tips and a noticeable hump on one of the sides (Figure 3a, Supplementary Figure 2a, Supplementary Movie 1). A three dimensional reconstruction of the most abundant class of P173 oligomers (Supplementary Figure 1a) was also calculated to assess their structural organization. The resulting model calculated from 430 particles of this class revealed that these oligomers were dodecamers comprised of six pairs of dimers aligned as a double-ring barrel (Figure 3b, Supplementary Figure 3a, Supplementary Movie 2) with the foot-like extensions connecting the dimer subunits and forming a rim in the equatorial plane of the structure while both poles show symmetrical openings. The oligomers were ~10 nm in diameter and ~7 nm high (Table 1).

We have also conducted single particle reconstructions from 558 images of rP172 monomers and 512 images of oligomers. The reconstructed model of the rP172 monomer closely resembles that of P173 (Figure 4a, Supplementary Figure 2b, Supplementary Movie 3). It has an overall ellipsoid shape with the characteristic foot-like process on one of the ends. However, the hump on the side of the monomer is not as apparent as in P173 (Supplementary Figure 4). The oligomer class of the same size as the P173 reconstruction

was selected (Supplementary Figure 1b). The overall structural organization of the rP172 oligomer closely resembles P173 with some minor differences (Figure 4b, Supplementary Figure 2b, Supplementary Movie 4). The size and aspect ratio of the oligomers are essentially the same (Table 1) and rP172 similarly appears as a dodecamer comprised of six pairs of dimers. At the same time there were some significant differences in the organization of the equatorial portion of the oligomers with the contact area between dimer subunits being broader in rP172 oligomers. Oligomers of rP172 also had wider polar openings than P173 oligomers, ~5 nm and ~4 nm in diameter respectively (Table 1).

Mineralization experiments in the presence of full-length native porcine amelogenin P173 revealed that small isolated mineral clusters were formed after 10 min (Figure 5a). The particles ranged between 1.14 to 1.68 nm in diameter with average particle size of 1.4 nm after defocus correction. A few small aggregates of 2–3 particles along with isolated particles appear after 30–60 minutes in the reaction, (Figure 5b,c). After 120 minutes, short linear chains of clusters have formed; together with random aggregates. Further examination of the 120 min sample revealed that in some cases linear chains nanoparticles have started to fuse together (Figure 5d). In the experiments with rP172 isolated mineral particles were observed after 10 min (Figure 6a) and large random aggregates of the clusters were observed at 30 (Figure 6b) and 60 min (not shown). Quite interestingly, after 2 hours of reaction these large random aggregates have become less frequent, while numerous linear chains of these clusters have appeared, and in many of these chains individual clusters started to fuse leading to the formation of needle-like elongated mineral particles (Figure 6c). In several instances parallel arrays of these elongated mineral particles were observed after 120 min as well (Figure 6d).

Discussion

The results of our cryo-TEM study of self-assembly of porcine amelogenins rP172 and P173 demonstrate major similarities between these proteins and murine amelogenin (Fang et al., 2011a). Specifically, both murine and porcine amelogenins undergo stepwise assembly in which cage- or barrel-like oligomers of several different sizes form and eventually they assemble into nanospheres. These results suggest that general mechanisms of amelogenin self-assembly are similar across different mammalian species. This is not surprising since amelogenin sequences, especially ones that are involved in protein-protein interactions, i.e. TRAP and C-terminals, are conserved (Paine and Snead, 1997; Paine et al., 2000; Toyosawa et al., 1998; Wang et al., 2006).

Single particle reconstructions of monomers of P173 and rP172 were remarkably similar to each other and to the previously published reconstruction of rM179. All three monomers had an ellipsoid shape and a foot-like process at one end. Our earlier studies of monomers of full length and truncated recombinant mouse amelogenins rM179 and rM166 (Fang et al., 2011a) demonstrated that the foot-like process consists of the C-terminal telopeptide. Since the C-terminal domain is conserved among mammalian species it is likely that the foot-like process in the porcine monomers also contains the C-terminal telopeptide (Toyosawa et al., 1998). At the same time we have found some subtle differences in the appearance of P173 and rP172 models. Specifically, P173 has a profound hump on one of its sides which appears to be somewhat diminished in rP172. We surmise that this difference might be due to the lack of a single phosphate group at S16 in rP172. Importantly this protrusion is similarly absent in murine recombinant amelogenin rM179 (Fang et al., 2011a).

Single particle reconstruction analysis has also revealed that the most abundant class of oligomers in both P173 and rP172 is dodecamer and that their structural organization closely resembles previously reported barrel-like dodecamers of murine recombinant rM179 (Fang

et al., 2011a). The reconstructed dodecamers of murine and porcine amelogenins had similar outer diameters, however rM179 dodecamer was higher, and its inner diameter was greater (Table 1). Our results further support the notion that the amelogenin self-assembly mechanism is similar among different species and that dodecamer is the most optimal oligomer packing. At the same time there are some differences between P173 and rP172 oligomers, which might be of importance to their function in the mineralization process. The electron density in recombinant amelogenins rP172 and rM179 (Fang et al., 2011a) is shifted toward the equator, with both of these structures having much wider openings at the poles, making them more barrel-like. In contrast, electron density in the P173 model is shifted toward the poles; these structures have much narrower polar holes, making them more cage-like. These differences might have profound effects on the ability of these oligomers to stabilize initial mineral clusters and structurally organize them.

The ability of full-length amelogenin to stabilize the initial mineral clusters and guide their assembly into parallel arrays followed by their fusion into needle shaped mineral particles has been previously proposed (Deshpande et al., 2010; Kwak et al., 2009) and later demonstrated by cryoTEM of calcium phosphate mineralization in the presence of rM179 (Fang et al., 2011a). Although both rP172 and P173 were able to stabilize mineral clusters for extended periods of time, P173 exhibited stronger stabilizing properties. These results are in perfect agreement with earlier studies of native and recombinant porcine amelogenins, which show that while rP172 could only temporarily prevent crystalline mineral formation, P173 was able to prevent crystallization almost indefinitely under comparable experimental conditions (Kwak et al., 2009; Kwak et al., 2011). Although rP172 could initially stabilize mineral clusters, these clusters soon started to come together and form aggregates. These aggregates were initially random but eventually transformed into chains of mineral clusters which in turn assembled into arrays, in which clusters start to fuse and form elongated nanoparticles. Such parallel arrays of elongated mineral nanoparticles are a hallmark of early enamel mineralization (Beniash et al., 2009); they comprise the building blocks of enamel-enamel rods. We have previously observed a similar sequence of mineralization in the presence of recombinant murine amelogenin rM179 (Fang et al., 2011a). Overall, the findings reported here provide further insight into the ability of amelogenin to control shape and organization of mineral particles and support the notion that the mechanisms regulating enamel biomineralization by amelogenin are universal across mammalian species.

One of the questions remaining is the nature of interactions between mineral clusters and amelogenin oligomers. Originally, the concept of small mineral clusters with stoichiometry $\text{Ca}_2(\text{PO}_4)_6$ and defined structure, that represent basic building blocks of ACP and orthophosphates has been proposed by Posner and coworkers (Betts and Posner, 1974); these clusters were later named Posner clusters. In recent years, a number of studies have been conducted to elucidate the chemical composition, structure and possible roles of mineral clusters in calcium phosphate mineralization in both biological and abiotic systems (Dey et al., 2010; Fang et al., 2011a; Habraken et al., 2013; Wang et al., 2012). Dey et al. observed in cryoTEM ~ 1 nm clusters as a first step of calcium phosphate mineralization (Dey et al., 2010) and interpreted them as Posner clusters. However, later more detailed studies of early steps of calcium phosphate mineralization in aqueous solution revealed that the prenucleation clusters are planar $[\text{Ca}(\text{HPO}_4)_3]^{4-}$ complexes, that transform into, so called, postnucleation clusters $[\text{Ca}_2(\text{HPO}_4)_3]^{2-}$ upon formation of ACP aggregates (Habraken et al., 2013). At this point, we do not have definitive data regarding the exact stoichiometry of the mineral clusters in our system, however based on the size of the particles, these are likely postnucleation clusters. If these are indeed the postnucleation clusters, our findings suggest that self-assemblies of full-length amelogenin have the capacity to prevent polymerization of mineral clusters for extended periods of time and can arrange them into parallel arrays of chain like structures prior to their fusion and

crystallization. At this point we can only surmise as what are exact relationships between the mineral clusters and protein assemblies. One possibility is that the prenucleation clusters enter the protein cage and form ionic or hydrogen bonds with charged amino acids of C-termini concentrated in the equatorial rim of the oligomers and phosphoserines in case of P173.

Our present findings on P173 are in agreement with earlier studies (Kwak et al., 2009; Kwak et al., 2011; Wiedemann et al., 2011) which have clearly demonstrated that native porcine amelogenins are strong inhibitors of crystallization, through their ability to stabilize ACP and prevent phase transformation, in comparison to their non-phosphorylated counterparts. The ability of P173 to stabilize the amorphous phase might be related to the higher negative charge of P173-mineral cluster complexes seen here, which could prevent fusion of oligomers due to the electrostatic repulsion. The single phosphoserine group in native amelogenin could also have a functional influence on amelogenin conformation, as we have recently found using phosphorylated and non-phosphorylated forms of leucine-rich amelogenin peptide (LRAP) (Le Norcy et al., 2011). Such effects on amelogenin conformation could significantly influence protein-mineral interactions and the ability of amelogenin to affect mineralization. The ability of P173 to stabilize ACP *in vitro* for long periods of time is also consistent with the recent demonstration that newly formed enamel mineral ribbons during the secretory stage of amelogenesis are comprised of an amorphous mineral phase that later transform into apatitic crystals (Beniash et al., 2009). These collective observations bring the question of how this transformation takes place *in vivo*. There are a few possible scenarios. It is possible that the inhibition of mineral phase transformation could be removed through selective proteolysis of the full-length amelogenin or through its dephosphorylation, although such possibilities *in vivo* must be verified. Another possibility is that *in vivo* amelogenin does not act alone but rather as part of multi-protein complex with other enamel matrix proteins which might modulate effects of amelogenin on mineral formation. The idea of such complexes forming at the Tomes' processes surface and regulating the formation of the intricate enamel mineral structure has been recently introduced (Simmer et al., 2012) and several *in vitro* studies have shown that amelogenin can interact with other enamel proteins and these interactions can modulate mineralization process (Bouropoulos and Moradian-Oldak, 2004; Fan et al., 2009; Iijima et al., 2010; Yang et al., 2011). Additional studies, however, are needed to gain insight into possible synergistic relationships between different elements of the organic matrix in the regulation of enamel biomineralization, including the removal of the inhibitory activity of native amelogenin through enzymatic processing. Despite the fact that many questions remain unanswered, the present study reinforces the notion that full-length amelogenin plays a key role in the regulation of enamel mineral formation and organization.

In summary, our cryoTEM studies of native and recombinant porcine amelogenins have revealed that both proteins undergo step-wise hierarchical self-assembly. Taken together with earlier studies of self-assembly of murine amelogenins these data suggest that the hierarchical assembly is a common mode amelogenin assembly among mammalian species. At the same time several differences were observed between recombinant-nonphosphorylated and native-phosphorylated amelogenins, which suggest that the differences in their effects on mineralization might be attributed to the structural differences in their protein assemblies. Furthermore, we demonstrate that recombinant non-phosphorylated porcine amelogenin can transiently stabilize initial mineral clusters and organize them into arrays of linear chains, followed by the fusion of the clusters in the chains. Similar modes of regulation of mineralization have been previously described for both murine and porcine recombinant amelogenins. In contrast, phosphorylated porcine amelogenin can stabilize initial mineral clusters and prevent their transformation. We propose that the enhanced capacity of phosphorylated porcine amelogenin to inhibit phase

transformation might be due, in part, to the effect of phosphorylation on the structural organization of its oligomers and charge distribution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

CryoTEM micrographs of assembly of P173 at 1 (a) and 10 (b) minutes in the reaction and several particle classes identified in the sample, including monomers (c); several classes of oligomers of different sizes (d–f), pairs of oligomers (g) and nanospheres (h).

Figure 2.

CryoTEM micrographs of assembly of rP172 at 1 (a) and 10 (b) minutes in the reaction and several particle classes identified in the sample, including monomers (c); several classes of oligomers of different sizes (d–f), pairs and small groups of oligomers (g) and nanospheres (h).

Figure 3. Single particle reconstructions of P173 monomer (a) and oligomer (b); asterisk identifies a foot-like process, double asterisk identifies a hump on the side of the monomer.

Figure 4. Single particle reconstructions of rP172 monomer (a) and oligomer (b); asterisk identifies a foot-like process, double asterisk identifies a hump on the side of the monomer.

Figure 5. CryoTEM micrographs of mineralization reaction in the presence of P173 at 10 min (**a**), 30 min (**b**), 60 min (**c**) and 120 min (**d**).

Figure 6.
CryoTEM micrographs of mineralization reaction in the presence of P173 at 10 min (**a**), 60 min (**b**) and 120 min (**c–d**).

Table 1

Dimensions of amelogenin oligomers in nm, obtained from the single particle reconstructions.

	OD	H	ID
P173	10	7	4
rP172	10	7	5
rM179 [*]	10	10	5.5

^{*} from Fang et al., 2011a

OD- outer diameter; ID- inner diameter; H- height