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pH Triggered Self-Assembly of Native and Recombinant Amelogenins under Physiological pH and Temperature *in vitro*

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Abstract

Self-assembly of the extracellular matrix protein amelogenin is believed to play an essential role in regulating the growth and organization of enamel crystals during enamel formation. This study examines the effect of temperature and pH on amelogenin self-assembly under physiological pH conditions *in vitro*, using dynamic light scattering, turbidity measurements, and transmission electron microscopy. Full-length recombinant amelogenins from mouse (rM179) and pig (rP172) were investigated, along with proteolytic cleavage products (rM166 and native P148) lacking the hydrophilic C-terminus of parent molecules. Results indicated that the self-assembly of full-length amelogenin is primarily triggered by pH in the temperature range from 13°C to 37°C and not by temperature. Furthermore, very large assemblies of all proteins studied formed through the rearrangement of similarly sized nanospherical particles, although at different pH values: pH 7.7 (P148), pH 7.5 (rM166), pH 7.2 (rP172), and pH 7.2 (rM179). Structural differences were also observed. The full-length molecules formed apparently tightly connected elongated, high-aspect ratio assemblies comprised of small spheres, while the amelogenin cleavage products appeared as loosely associated spherical particles, suggesting that the hydrophilic C-terminus plays an essential role in higher-order amelogenin assembly. Hence, tightly controlled pH values during secretory amelogenesis may serve to regulate the functions of both full-length and cleaved amelogenins.

Keywords

Enamel; Biomineralization; Amelogenin; Self-assembly; Dynamic Light Scattering; Turbidity

1. Introduction

Tooth enamel represents an intriguing biomineralization system due to its high mineral content, outstanding mechanical properties and, in contrast to bone, because it does not undergo remodeling after mineralization is complete. The hierarchical microstructure of enamel is comprised of bundles (rods) of extremely long parallel arrays of calcium phosphate crystals (Daculsi et al. 1984) that form an intricate interwoven structure with traces of organic matter between the rods. This makes tooth enamel as hard as titanium without being brittle (Habelitz et al. 2001; Marshall, Jr. et al. 2001). Tooth enamel formation is regulated by ameloblasts.

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During early stages of formation (secretory stage), enamel consists of very small ribbon-like crystals embedded within a protein gel matrix. The spatial organization of these crystals is similar to the crystalline arrangement in the mature enamel, however, they amount to only around 30 % w/w during the secretory stage, whereas in mature enamel the mineral comprises more than 95 % w/w, based on detailed analyses of developing rat enamel (Smith, 1998).

It has been suggested that both crystal shape and arrangement in forming enamel are controlled via a cooperative mechanism of simultaneous protein assembly and crystal growth (Beniash et al. 2005; Margolis et al. 2006). Amelogenins constitute up to 90% of the extracellular protein matrix in developing enamel, and several studies have confirmed that amelogenin is essential for proper enamel mineral formation *in vivo* (Lyngstadaas et al. 1995; Gibson et al. 2001). Yet, the exact role of enamel matrix proteins and the mechanisms controlling mineralization and crystal organization during enamel formation are not fully understood. Although this area has been and continues to be the focus of intense research, the specific roles of the different enamel matrix proteins found *in vivo* have not been fully elucidated. However, a combination of *in vivo* (Snead et al. 1998; Paine et al. 1998; Wright et al. 2002; Gibson et al. 1997; Gibson et al. 2001; Gibson et al. 2005) and *in vitro* approaches using native and recombinant matrix proteins, have provided invaluable insights. In particular, it has been shown that amelogenin self-assembles *in vitro* to form nanospheres (Fincham et al. 1994; Fincham et al. 1995; Moradian-Oldak et al. 1998a; Moradian-Oldak et al. 2000). Moreover, it has been suggested that these nanospheres further aggregate into higher-order structures that guide crystal growth and organization (Moradian-Oldak et al. 1994; Moradian-Oldak et al. 1995; Fincham et al. 2000; Aichmayer et al. 2005; Beniash et al. 2005; Du et al. 2005). Available data suggest that these aggregation processes are pH dependent (Moradian-Oldak et al. 1994; Simmer and Fincham 1995; Moradian-Oldak et al. 1998a; Fincham et al. 1998; Aichmayer et al. 2005; Beniash et al. 2005; Habelitz et al. 2005; Margolis et al. 2006). Whereas some pH variability between acidic (below pH 6) to neutral conditions has been shown during maturation stage amelogenesis (when the organic matrix is proteolytically removed and the initial crystals grow substantially in thickness and in width), the pH during the secretory stage of amelogenesis is tightly regulated between pH 7.2 to 7.4 (Smith 1998; Smith et al. 2005; Smith et al. 1996; Sasaki et al. 1991). A major challenge in working with enamel matrix proteins *in vitro* is that their solubility under the latter physiological pH conditions is very low (Tan et al. 1998; Fincham et al. 1994; Moradian-Oldak et al. 1994). For this reason, few *in vitro* studies have been carried out under these conditions. In contrast, the literature on amelogenin studied under conditions of higher (pH $\geq$ 8) and lower pH (pH $<$ 6) is abundant (Moradian-Oldak et al. 1994; Moradian-Oldak et al. 1995; Fincham et al. 1995; Fincham and Moradian-Oldak 1995; Fincham and Simmer 1997; Moradian-Oldak et al. 1998a; Moradian-Oldak et al. 2000; Moradian-Oldak et al. 2001; Diekwisch 1998).

Using a transgenic mouse model, amelogenin self-assembly has also been shown to be regulated by specific domains within highly conserved N- and C-terminal regions of amelogenin that play essential roles in proper enamel formation (Snead et al. 1998; Paine et al. 2000). As shown in Fig. 1, the full-length amelogenins from pig and mouse consist of three domains, a tyrosine-rich N-terminal domain of 45 amino acids (referred to as tyrosine rich amelogenin protein, TRAP), a hydrophobic central domain, and a hydrophilic C-terminal domain (Snead et al. 1985; Hu et al. 1996). The N-terminal and C-terminal segments have also been shown to play important roles in amelogenin self-assembly *in vitro* (Moradian-Oldak et al. 2000). Both *in vivo* and *in vitro* studies have provided insight into the importance of the modular nature of amelogenin and its relevance to proper protein assembly and enamel formation (Paine and Snead 1997; Paine et al. 2000; Moradian-Oldak et al. 2000). Such findings led to the speculation (Paine et al. 2000) that a transient tethering of a lectin-like binding domain in the N-terminus of amelogenin to the glycocalyx of the retreating ameloblast may also help align amelogenin nanospheres and guide enamel mineral growth. However, little is known

about the underlying mechanisms of protein assembly, although amelogenin nanospheres were first described more than a decade ago (Robinson et al. 1981; Fincham et al. 1994). Recently, we proposed a model for amelogenin assembly based on small angle X-ray scattering (SAXS) and dynamic light scattering (DLS) analyses (Aichmayer et al. 2005), suggesting that the amelogenin nanospheres consist of a hydrophobic and electron dense core that is surrounded by a less electron dense and hydrophilic corona comprised of the charged C-terminus. Data from that study also suggested that ‘nanospheres’ of the full-length amelogenin have the ability to assemble further to form extended, perhaps, chain-like structures in solution, consistent with other *in vitro* data obtained under specified conditions (Habelitz et al. 2004; Beniash et al. 2005; Du et al. 2005).

The present study uses recombinant and native amelogenins, DLS, turbidity, and TEM techniques to obtain further mechanistic insight into the role of amelogenin assembly in enamel formation. More specifically, our studies have focused on the role of the C-terminal hydrophilic portion of amelogenin (Fig. 1), first described by cDNA cloning (Snead et al. 1985), which is proteolytically removed soon after the secretion of the full-length parent molecule into the extra-cellular space. Importantly, it has been reported (Shaw et al. 2004), based on solid state NMR data, that the C-terminus region of amelogenin interacts with hydroxyapatite crystal surfaces. This suggestion is consistent with previously reported results that show that the hydrophilic C-terminus of amelogenin promotes amelogenin binding to hydroxyapatite surfaces and enhances the inhibition of hydroxyapatite seeded crystal growth (Aoba et al. 1987; Aoba and Moreno 1991; Moradian-Oldak et al. 1998b). We believe that a better understanding of the amelogenin assembly process under physiological pH conditions will provide insight into mechanisms of enamel formation and its unique structure and function (Rensberger 2000; Popowics et al. 2004).

2. Materials and methods

2.1. Protein Preparation

Recombinant amelogenins from mouse (rM179, rM166) and pig (rP172) were used in this study, along with a native pig amelogenin cleavage product (P148). As illustrated in Fig. 1, the shorter molecules rM166 and P148 differ from the full-length molecules rM179 and rP172, respectively, as they both lack the hydrophilic C-terminal tail of 13 amino acids. The native P148 is also phosphorylated at serine-16, contains an N-terminal methionine, and lacks 12 C-terminal amino acids in addition to the hydrophilic C-terminal tail. P148 was selected for study, in part, because it is the most abundant protein found in developing pig enamel (Fincham and Moradian-Oldak 1996; Yamakoshi et al. 1994).

Recombinant amelogenins from mouse (rM179 and rM166) and pig (rP172) were produced and purified as previously described (Simmer et al. 1994). These recombinant amelogenins, derived from bacteria, lack the single phosphate group at serine-16 and the N-terminal methionine, present in the native proteins. The native pig amelogenin P148 was produced and purified as previously described (Yamakoshi et al. 2003).

2.2. Protein solution preparation

Protein stock solutions (5 mg/mL) were prepared by dissolving lyophilized protein in distilled de-ionized water (DDW). Stock solutions were stored for 24 hours at 4°C to facilitate complete solubilization. These solutions were used for the preparation of sample solutions with a final concentration of 2mg/mL. DDW, as well as all buffer stock solutions, were filtered (0.22 µm) before use. Stock solutions of MOPS buffer (Sigma Aldrich) were prepared at room temperature without pH adjustment. This solution had a pH of 3.7 and was used to assess the effect of increased ionic strength at low pH. MOPS buffer has a small temperature coefficient

($-0.006 \Delta\text{pH}/^\circ\text{C}$) (Mohan 2003). TRIS/HCl (Sigma Aldrich) stock solutions were prepared and adjusted to have desired pH values at 13°C , 25°C or 37°C . TRIS/HCl has a large temperature coefficient ($-0.031\Delta\text{pH}/^\circ\text{C}$). TRIS/HCl buffer was used to study protein assembly over a pH range between pH 8.3 and pH 7.0 by varying the temperature during the course of the experiment. TRIS/HCl buffer also decreases 0.4 pH units with 100-fold dilution. Care was therefore taken to ensure that the targeted pH values were actually achieved for the sample preparation and that dilution effects were minimal (± 0.01 pH units).

2.3. Sample preparation for dynamic light scattering (DLS) experiments

Samples (30 μL) of proteins dissolved in DDW were centrifuged ($10,900 \times g$) for 20 minutes at 4°C , just prior to all DLS experiments. Filtered pH-adjusted TRIS/HCl or MOPS buffers kept on ice were then added to the protein solutions (final buffer concentration of 80 mM) to yield the desired pH value. More specifically, 80 mM TRIS/HCl buffers adjusted to have pH 7.2 at 13°C (Buffer I), 25°C (Buffer II), and 37°C (Buffer III) were used to determine the influence of pH and temperature on amelogenin assembly. Initial DLS experiments were performed at protein concentrations of 0.2 mg/mL, 1mg/mL, and 2mg/mL, to test for concentration effects on particle size, which were found to be negligible in pH-adjusted samples. The scattering intensity was very low in samples of 1 mg/mL and 0.2 mg/mL of protein in DDW without buffer, but reliable results were obtained at protein concentrations of 2mg/mL under these conditions. Hence, all data reported here are derived from experiments using 2mg/mL protein.

2.4. Sample preparation for turbidity measurements

pH-adjusted protein solutions were prepared in the same manner as for DLS analyses, however, using a final sample volume of 80 μL . Seventy (70) μL of this sample were placed into the pre-cooled quartz micro-cell before it was inserted into the temperature-controlled cell holder for turbidity experiments.

2.5. Dynamic Light Scattering (DLS)

2.5.1 DLS Instrumentation—Temperature-dependent measurements were performed using a commercially available DLS instrument (DynaPro MSXTC/ 12) with a gallium-arsenite diode laser (DynaPro-99-E-50) of 825.2 nm emission and programmable power. This instrument is equipped with a temperature-controlled housing (precision of 0.1°C). A quartz cuvette of 12 μL sample volume was used in all experiments. Scattering data were collected at an angle of $\theta = 90^\circ$ and processed using the software program DYNAMICS V6, version 6.3.40. A proprietary non-negative least square algorithm and cumulants were used in the calculation of mean particle size values, percent polydispersity, the baseline value, and the sum of squares. Each measurement consisted of 20 acquisitions of 5 seconds each. A regularization algorithm was used to resolve for up to five multimodal populations and their polydispersity, normalized to the mean size of the peak and the hydrodynamic radius (R_H). R_H is an indirect measure of particle size, since it is based on the Brownian motion of particles surrounded by a hydration layer in dilute solution (Berne and Pecora 2000). The DYNAMICS V6 software also provides information about the molecular weight of the particles, the relative amount of light that is scattered by each population, and the estimated value for the relative mass percentage of each population. Since DLS is a method that is very sensitive to large particle sizes, the presence of very low levels of either a contaminant (*e.g.*, a dust particle) or a large protein aggregate, will skew the data towards larger particle sizes. To avoid this potential problem of overestimating particle sizes, especially at the onset of large particle formation, all R_H and polydispersity data reported here result from mass weighted calculations, which take this factor into account. All data were modeled as isotropic spheres.

2.5.2 DLS data collection—A 12 μL aliquot of protein solution prepared on ice was transferred to the quartz cell maintained at 4°C in the DLS instrument and covered with one drop of mineral oil to prevent evaporation. The quartz cell was then closed with a plastic stopper and sealed with parafilm. Measurements were started 20 minutes after inserting the quartz cell into the temperature-controlled chamber. The temperature was increased in three-degree steps to 43°C. Samples were allowed to equilibrate for 5 minutes at each temperature before DLS data were collected. Measurements consisted of 20 acquisitions of 5 seconds each and were repeated three times at the same temperature at 5-minute intervals. Consequently, each sample was kept at any given temperature for slightly more than 20 minutes. This approach allowed us to detect whether particle size changes occurred during data collection at each temperature.

Collected data were included for further analyses based on the quality of the autocorrelation curve. The data were considered good when the auto-correlation curve was (1) continuous, (2) smooth, (3) the intensity autocorrelation started at 1.5 or greater, and (4) the baseline, i.e. the measured value of the last coefficient in the autocorrelation curve, was 1.000 ± 0.005 . The accuracy and precision of the DLS measurements for small particle sizes was determined by repeated measurements of lysozyme (from egg white, Sigma Aldrich) solution in $5 \times \text{PBS}$ buffer (pH 6.8), which yielded a mean R_H value of 1.8 nm (0.1 nm s.d.). This value is in excellent agreement with the calculated radius of 1.56 nm of the protein modeled as a hard sphere and a single hydration layer, which extends the radius by 0.28 nm.

Based on results from earlier studies (Nikiforuk and Simmons 1965), it was anticipated that amelogenin samples would form an opaque gel at room temperature under certain conditions. However, since in a gel the movement of the gel-forming particles is minimal (Brinker and Scherer 1990), it is not possible to obtain meaningful DLS data under such conditions, as DLS measures diffusion of particles in solution (Berne and Pecora 2000). When a sample forms a gel, the intensity of the scattered light increases orders of magnitude as a result of the decrease in free particle movement (Berne and Pecora 2000). Therefore, the formation of very large aggregates or a gel was indicated by a jump in the signal intensity to values above the detection limit of the instrument. When this was observed, the sample cuvette was removed and inspected by eye. The relative transparency or opacity of the sample was noted before the experiment was continued.

2.6. Turbidity Measurements

Turbidity measurements of protein solutions prepared similarly to those used in DLS studies were made using a Genesys 5 UV-VIS Spectrophotometer (ThermoElectron, Waltham, MA), equipped with a water-jacketed cell holder (ThermoElectron, Waltham, MA) for temperature control, by observing the change in optical density (OD) of the samples at 595 nm. The temperature was regulated using a circulating water bath (Isotemp 3016D, Fisher Scientific) with a temperature control range between 7 °C and 60 °C (± 0.1 °C). The temperature in the sample quartz cell inserted in the spectrophotometer was monitored using a Dual Thermometer, Type K (Fisher Scientific, ± 0.1 °C precision) inserted into a blank quartz cell, adjacent to a the sample cell, filled with buffer solution of equal volume and concentration as in the sample.

2.6.1. Protocol for turbidity experiments—Protein solutions were prepared on ice as described above. At the start of each experiment, the control cell containing buffer solution was inserted into the cell holder, which was kept at 37°C. The buffer absorbance was measured until a constant value was obtained when thermal equilibrium was attained. The difference in absorbance between cold buffer solution and buffer solution at 37 °C was less than 0.010 A. Seventy (70) μL of the cold protein sample were then placed into a pre-cooled quartz micro-cell. The sample cell was then inserted into the cell holder and data collection was started immediately in 5-second intervals, as the OD increased and reached a maximum within a very

short time period. When a lower rate of change was observed during a subsequent decline in OD, data collection intervals were increased to 10, 20, and 30 seconds and after 1 hour to intervals of several minutes. The sample was kept at a constant temperature of 37°C until the OD dropped below 0.500 A. Changes in OD were subsequently plotted as a function of time.

2.7 TEM analyses

Eighty five (85) μ L samples of protein solutions (2 mg/mL) in TRIS/HCl buffer for TEM were prepared as described above. Duplicate samples for TEM were prepared by placing carbon coated Ni-grids #400 (EMS, Hartfield, PA) on top of 40 μ L droplets of the protein solution. Samples were then incubated in a humidity chamber at 37°C for 20 minutes (i.e., shortly after a peak in OD was observed in turbidity experiments) and 150 minutes (i.e., when in turbidity experiments the OD had decreased and was nearly at a plateau). After incubation, the TEM grids were blotted on qualitative #2 Whatman filter paper and rinsed in filtered DDW. The grids were then air-dried for 30 to 60 seconds and negatively stained with 1% filtered phosphotungstic acid (pH 7.2) for 30 seconds. After 30 seconds, the grids were blotted again and air-dried. TEM analyses were carried out using a JEOL 1200 TEM microscope at 100 KV, in bright field. Images were captured using an AMT CCD camera (AMT, Danvers, MA). Image analyses (i.e., particle size measurements) were subsequently performed using ImageJ 1.36 (NIH, Bethesda, MA, <http://rsb.info.nih.gov/ij/download.html>). The measurement data were exported into Microsoft Excel (Windows Office XP) for further processing.

2.8. Statistical Methods

Single factor ANOVA tests (alpha 0.01) were used to compare the results derived from TEM analyses and to test for significant differences between the size of the assemblies formed in samples of the cleaved and full-length amelogenins at the 20 and 150 minute incubation time points.

3. Results

3.1 Effect of pH and temperature on amelogenin self-assembly – DLS findings

3.1.1 Amelogenins at low pH—Mouse and pig amelogenins purified according to the described procedure had pH values between pH 3.1 and pH 3.5 when dissolved in DDW at concentrations between 2 and 5 mg/mL. Under these acidic conditions, the proteins exist as monomers or dimers, based on DLS measurements shown in Table 1. The data in Table 1 summarize mean R_H values and standard deviation for the full-length amelogenins and cleavage products over the temperature range from 4°C to 43°C in DDW and in a 80mM MOPS buffer at pH 3.7. As indicated by the small variability in all cases, the particle sizes did not change in DDW or in MOPS buffer over the wide temperature range studied.

The calculated radius for amelogenin monomeric spheres is about 1.8 nm (Table 1), based on the average (0.73 cm³/g) experimentally determined partial specific volumes for soluble, globular proteins (Harpaz et al. 1994). The measured R_H values between 1nm and 2 nm for amelogenins in DDW (pH ~ 3.5) or in 80 mM MOPS buffer (pH 3.7) indicate that both the full-length and cleaved amelogenins studied are present as monomers or dimers in solution under these acidic conditions over a wide temperature range from 4 °C to 43 °C.

3.1.2. Influence of temperature-triggered pH changes on amelogenin assembly in TRIS/HCl buffered samples—TRIS/HCl buffers adjusted to specific pH values at different temperatures were carefully examined to ensure that the desired pH value at a given temperature would be attained when the buffers are mixed with the protein samples. As summarized in Table 2, the pH of protein samples very closely matched the pH of control buffer solutions that did not contain protein. The data clearly indicate a decrease in pH upon

temperature increase (Table 2), consistent with the large temperature coefficient for this buffer (Mohan 2003). Importantly, the pH data show that samples prepared with TRIS/HCl buffer adjusted to pH 8.0 at 25°C reach lower pH values when the temperature is raised to 37°C (Table 2). The pH values of control and protein samples (Table 2, shown in bold) were very close when measured at the same temperature at which the pH adjustment was carried out.

Taking advantage of the marked influence of temperature on the pH of TRIS/HCl buffers, studies were carried out to test the hypothesis that amelogenin self-assembly is triggered by pH rather than by temperature.

As illustrated in Fig. 2, R_H values of approximately 13 nm were observed for rP172 samples as the pH was reduced from higher pH values to values slightly greater than pH 7.2 by increasing the sample temperature for the three buffers examined, as shown. R_H values then increased sharply and the scattering intensity went off scale at approximately the temperature at which the pH of the TRIS/HCl was adjusted to pH 7.20. As shown (Fig. 2b), in samples with TRIS/HCl buffer II adjusted to pH 7.20 at 25°C, the DLS scattering intensity went off scale at 25°C, within a short equilibration time of only a few minutes. In contrast, in samples with TRIS/HCl buffer III adjusted to pH 7.20 at 37°C (Fig. 2a), the particle size did not change at 25°C but remained nearly constant until about 30°C, and changed dramatically at 37°C where scattering intensity went off scale within five minutes. Similarly, in samples with TRIS/HCl buffer I adjusted to pH 7.20 at 13°C (Fig. 2c), the DLS scattering intensity went off scale at ~13°C. Consistent with the dramatic increase in R_H , the protein solutions became opaque based on visual inspection.

A significant increase in the polydispersity of rP172 particle sizes was also observed immediately prior to the scattering going off scale at 25°C and 37°C. This is reflected by the large standard deviation in R_H values at these points (Figs. 2a,b), whereas the standard deviation and polydispersity was very small at lower temperatures (and higher pH values). This increase in standard deviation resulted from the formation of two populations of different particle sizes (data not shown). Typically one of these groups consisted of particles with R_H values of hundreds of nanometers, whereas the other population consisted of particles with R_H values of 4 to 8 nm comprising up to 50% by mass. The particles in the latter population were smaller than the typical particles sizes (R_H ~13 nm) observed at lower temperatures, yet larger than those observed under acidic conditions (Table 1). Samples of rM179 also clearly showed a population of small particles with R_H values of 4 nm just prior to an increase in opacity and off-scale scattering when pH 7.2 was reached at 37°C (data not shown). A population of small particles of rM179, however, was not observed at this transition point when pH 7.2 was reached at 25°C (data not shown).

3.1.3 Influence of temperature-triggered pH changes on the formation of higher-order assemblies of full-length and cleaved amelogenins—As shown in Fig. 3, the full-length amelogenins (rP172 and rM179) and cleavage products (rM166 and P148) exhibited similar R_H values of 13 nm from approximately pH 8.0 to pH 7.8. As illustrated, the gradual increase in temperature led to a pH drop and a marked increase in particle size for all proteins studied at specific temperatures and associated pH values, as indicated by a sharp increase in light scattering intensity to a point where the signal went off scale. rM179 was found to behave very similarly to rP172 where the sharp increase in R_H was observed at approximately pH 7.2. Under these conditions, the sample appeared opaque upon visual examination. Samples of P148 and rM166 also exhibited a marked increase in R_H and became opaque but at significantly higher pH values of pH 7.7 and pH 7.5, respectively. In contrast, samples of both full-length molecules, rP172 and rM179, did not form larger assemblies or turn opaque at these higher pH values. Interestingly, unlike the full-length amelogenins (as seen for rP172 in Fig. 2), a marked

increase in polydispersity was not observed immediately preceding the off-scale scattering in experiments with the cleavage products P148 and rM166 (data not shown).

3.1.4. Structural organization of large assemblies of amelogenin: Turbidity and TEM Results—

TEM and turbidity experiments were carried out to compare protein assemblies formed by the full-length and cleaved amelogenins at pH 7.2 at 37°C. These pH conditions closely represent those found in secretory enamel (Aoba and Moreno 1987; Smith et al. 1996) and found here to trigger a marked increase in the particle size of the full-length amelogenins, as described above. The turbidity results obtained are shown in Fig. 4 and illustrate that solutions of all four proteins, rM179, rP172, rM166, and P148 exhibit a sharp and marked increase in OD (due to light scattering at 595 nm), immediately upon transfer from 4°C (pH 8.2) to 37°C (pH 7.2). After this dramatic increase, the OD decreases in all four proteins, albeit at different rates. This decrease in turbidity is likely caused by sedimentation of protein aggregates, as confirmed by a marked reduction in absorbance in the samples at 280 nm (data not shown), and not due to a disassembly of protein aggregates. Consistent with the observed separation of the protein aggregate from solution, a white layer of sediment was observed on the bottom of the sample cuvette after 150 min for all 4 proteins. The different rates of change in turbidity seen in Fig. 4 may suggest differences in structural and chemical properties of each protein aggregate.

TEM experiments were performed to confirm some of these observations and to examine the structural features of the formed assemblies. Samples for TEM were collected after 20 min. (peak of turbidity change) and 150 min. (after the OD of the sample reached a plateau) of sample equilibration at 37°C, as described in Materials and methods. Particle dimensions were obtained by direct measurement of TEM micrographs and are summarized in Table 3. Typical TEM results obtained for P148 and rP172 at both sampling times are shown in Fig. 5 and Fig. 6, respectively. During the initial stages of assembly (examined at 20 min.), both the full-length rP172 and its cleavage product P148 were found to predominantly form spheres of similar size, 11 ± 2.5 nm in diameter. Similarly sized nanospheres were also observed for rM179 (10 ± 2.5 nm) and rM166 (11 ± 2.4 nm) at 20 min. Hence, the particle sizes were essentially the same for all proteins examined at 20 min. However, after 150 minutes incubation at 37°C, significant differences were observed. As shown in Fig 5b and Table 3, at 150 min, isolated spheres of P148 were again observed, although they were almost twice the diameter (20 ± 4.4 nm) of the P148 spheres observed at 20 min. The difference in particle size between 20 minutes and 150 minutes incubation was statistically significant ($p < 0.001$). As shown in Table 3, statistically significant changes ($p < 0.001$) in nanosphere diameter were also observed in the rM166 samples, however, these nanospheres were slightly (~20%) smaller after 150 minutes of incubation in comparison to those observed at 20 minutes, with an average diameter of 9 nm. Thus, the predominant structure observed for the amelogenin cleavage products examined was loosely associated nanospheres, after 150 min.

After 20 min of incubation, the full-length molecules (rP172, rM179) were similarly found to predominantly form nanospheres that were indistinguishable in size (10 – 11 nm) from those observed for the cleaved molecules (rM166, P148) at this point in time, as shown in Table 3 and in Fig. 6a for rP172. However, unlike the cleavage products, the number of isolated nanospheres of the full-length amelogenins observed after 20 minutes incubation (e.g., Fig. 6a) was dramatically reduced after 150 minutes of incubation, while chains consisting of 5 to 10 nanospheres became the predominant feature (Fig 6b). In rP172 samples, these chain-like structures, comprised of closely associated smaller spheres (7 ± 2.1 nm in width), had a mean length of 51 ± 26.2 nm and a mean aspect (L/W) ratio of 7.8 ± 4.8 (Table 3). Almost identical results were obtained for the full-length recombinant protein from mouse rM179 (Table 3).

Discussion

As noted in the Introduction, and confirmed by present results, *in vitro* studies clearly have shown that enamel matrix proteins exhibit the tendency to form nanospheres (*e.g.*, Fincham et al. 1994; Fincham et al. 1995; Moradian-Oldak et al. 1998a; Moradian-Oldak et al. 2000), as well as gel-like structures (Eastoe 1979; Wen et al. 1999). The importance of such assemblies with respect to enamel formation is based on TEM observations that suggest that filamentous (Travis and Glimcher 1964; Smales 1975) and spherical (Fearnhead 1965; Robinson et al. 1981; Fincham et al. 1995) nanometer-sized (5 - 100 nm) structures are associated with developing enamel mineral. The onset and extent of amelogenin aggregation has been reported to be dependent on both pH and temperature (Moradian-Oldak et al. 1998a; Petta et al. 2006). Recently, we reported that the full-length recombinant mouse amelogenin rM179 formed nanometer sized aggregates with R_H values of ~17 nm in TRIS/HCl buffer at pH 8.0, based on DLS measurements, while the temperature was slowly (over a period of more than 24 h) increased from 4 – 44°C (Aichmayer et al. 2005). Upon a further increase in temperature to 45 – 46°C, the R_H values increased sharply to ~57 nm suggesting the formation of larger assemblies. Subsequent measurements and analyses using a combination of SAXS and DLS findings suggested that the larger assemblies observed at 45 – 46°C were chain-like and comprised of nanometer sized spheres (Aichmayer et al. 2005). Consistent with these findings, a similar temperature-induced increase in R_H for rM179 in TRIS/HCl buffer at pH 8.0 was previously reported (Moradian-Oldak et al. 1998a), although this transition was observed at a lower temperature of 37°C (from 15-17 nm at 20°C to 58 nm at 37°C).

In the study by Aichmayer et al. (2005), however, we did not consider the influence of temperature on the pH of the TRIS/HCl buffer (adjusted to pH 8 at room temperature), as we have in the present study. Taking this factor into account, we would now estimate (see Table 1) that the noted transition at 45-46°C took place at a pH value (pH 7.55-7.52) that was approximately 0.5 pH units lower than the expected pH 8.0, and somewhat closer to that observed (~pH 7.2) in the present study. R_H values at this transition temperature were also shown (Aichmayer et al. 2005) to increase with time over a 130 min period, suggesting that kinetics can also play an important role in the assembly process. It should be noted, however, that the temperature-induced ~60 nm (by DLS) assemblies of rM179 observed in these previously reported studies (Moradian-Oldak et al. 1998a; Aichmayer et al. 2005) differ significantly from the very large assemblies observed in the present study at pH 7.2. These differences likely reflect the significant influence of small differences in pH on amelogenin assembly, observed in the present study, as well as the amount of time the protein was allowed to equilibrate under specified conditions. In addition, it has been recently reported, based on DLS measurements, that large gel-like aggregates form from a solution of pig enamel extracts, consisting of a mixture of amelogenins rather than the pure full-length amelogenins and cleavage products used in the present study, at pH 6 when the temperature is raised above 30°C (Petta et al. 2006). Although a consideration of all available data suggest that temperature may influence the onset, extent and even the nature of amelogenin assembly, results from the present study clearly show that the assembly of full-length amelogenin from both pig and mouse are tightly regulated by pH under conditions of physiological pH and temperature (*e.g.*, Fig. 2). Previously, it was reported that the effect of pH on the size of rM179 assemblies was more apparent at 37°C than at 25°C (Moradian-Oldak et al. 1998a), leading the authors to conclude that, under physiological conditions, local pH changes might play a critical role in controlling the structural organization of the enamel matrix. Although our (DLS) data do not necessarily support the findings of this earlier study, results from our present study provide direct evidence that amelogenin assembly is tightly regulated by pH rather than by temperature.

Our present findings also clearly indicate that, although both cleaved and full-length molecules form similarly sized nanospheres at or above pH 7.7 (Fig. 3), there are two major differences

between full-length and cleaved molecules regarding further self-assembly. First, the formation of larger assemblies occurs at lower pH values for the full-length amelogenins, compared to the cleaved amelogenins studied. Second, the full-length amelogenins form structurally different assemblies than the cleaved molecules at pH 7.2, i.e., under conditions that closely reflect the physiological pH of secretory enamel. The physiological pH of the extra-cellular enamel matrix during the secretory stage has been reported to be close to neutrality with average values of pH 7.26 ± 0.04 in pig (Aoba and Moreno 1987) and pH 7.23 ± 0.12 in rat (Smith 1998; Smith et al. 2005; Smith et al. 1996) enamel. Thus, our findings again suggest that the extent and nature of assembly, and consequently the biological functions of the full-length amelogenin and possibly different cleavage products in the enamel matrix, are regulated by tightly controlled pH values found in developing enamel.

Changes in pH can clearly affect protein self-assembly through the modification of the charge on amino acid side-chains. Such changes can enhance both intra- and inter-molecular protein interactions by reducing electrostatic repulsion. This is illustrated in a recent study on facilitating the self-assembly of charged proteins (Yu et al. 2006). Since intermolecular repulsion of proteins is minimal at their isoelectric pH, it is not surprising that both full-length and cleaved molecules form large assemblies at pH values that are close to their calculated isoelectric points (<http://www.scripps.edu/~cdputnam/protcalc.html>). The calculated isoelectric point is at pH 7.05 for the full-length amelogenins, pH 7.23 for rM166, and pH 7.94 for P148. Indeed, the differences in calculated isoelectric points are reflected in the general trend in the pH values at which a sharp increase in light scattering was observed for the four amelogenins studied (Fig. 3), indicating the formation of larger aggregate structures. Hence, electrostatic interactions clearly play a key role in promoting amelogenin self-assembly. Accordingly, ionic strength may also influence amelogenin assembly. Although, the ionic strength was similar for each buffer used in the present studies, it was lower than that reported for enamel fluid (Aoba and Moreno, 1987). Hence, this potentially important physiological factor was not considered in the present study. More importantly, however, our data show that the presence of the hydrophilic C-terminus has a marked effect on the structure of the formed assemblies, as described. Our findings are consistent with both *in vivo* studies, using a transgenic mouse model (Snead et al. 1998; Paine et al. 2000), and *in vitro* measurements, using amelogenin mutant molecules (Moradian-Oldak et al. 2000), that strongly support the notion that self-assembly *in vivo* is regulated by specific domains within highly conserved N- and C-terminal regions of amelogenin that play essential roles in proper enamel formation.

Our TEM observations show that at pH 7.2 the full-length amelogenins prevail at 150 minutes as elongated assemblies that are made up of chain-like, tightly connected small spheres of about 7 nm in diameter (Fig. 6b), whereas the cleaved amelogenins appear as spherical structures that are not tightly connected (Fig. 5b). The diameter of nanospheres in chains observed at 150 min for the full-length protein was significantly smaller than that observed at 20 min ($p < 0.001$) (Table 3). The reduction in particle size observed using TEM is consistent with our DLS findings (section 3.1.2) of a small population of particles prior to off-scale scattering and suggests that a reorganization of amelogenin nanoparticles occurs during the formation of the elongated structures. A change in particle size towards smaller particles of 4 to 8 nm R_H was observed in samples of rP172 and rM179 directly before the sample turned opaque and the DLS scattering intensity went off scale when a pH of 7.2 was reached at 37°C. These data clearly show that full-length amelogenin nanospheres undergo significant structural changes prior to further self-assembly to higher-order structures under physiological pH conditions at pH 7.2. Although structural parameters of protein assemblies cannot be precisely determined based solely on DLS data, these data together with the results of our previous studies (Aichmayer et al. 2005) strongly suggest that a rearrangement of amelogenin assemblies (nanospheres) takes place prior to the formation of apparently larger assemblies. In support of this general finding, it was previously shown using DLS that a mono-modal distribution of

rM179 nanospheres (15-17 nm) at pH 8 becomes bi-modal or multimodal above 27°C prior to the formation of larger (~60 nm) assemblies, leading these investigators to conclude that above a certain temperature rM179 refolds and assembles into a new conformation (Moradian-Oldak et al. 1998a).

In the present study, changes in nanosphere particle size were also observed by TEM with time during the equilibration of cleaved molecules, particularly P148. In contrast to the full-length molecule, which forms chains comprised of smaller particles at 150 minutes, P148 exhibited larger particles at 150 min of incubation. However, the opposite was observed for rM166. Hence, as in the case of the full-length amelogenins, the amelogenin cleavage products studied also appear to reorganize at critical pH values (Table 3, Fig. 5).

The frequently examined cleavage products rM166 and P148, used in the present study primarily lack the hydrophilic C-terminus (Fig 1). This segment of the full-length amelogenin has been reported (Shaw et al. 2004) to interact with hydroxyapatite crystal surfaces and, through comparative studies using full-length and cleaved amelogenins, to promote full-length amelogenin binding to hydroxyapatite surfaces (Aoba et al. 1987; Aoba and Moreno 1991; Moradian-Oldak et al. 1998b), resulting in an enhanced inhibition of seeded crystal growth. The C-terminus of amelogenin has also been shown to play an essential role in regulating both calcium phosphate (Beniash et al. 2005) and silica (Fowler et al. 2006) mineral organization *in vitro*. Thus, on the basis of these functional studies, it could be inferred that amelogenin cleavage products studied do not play critical roles in enamel mineral formation, despite the fact that they can constitute a significant portion of the developing enamel matrix (Yamakoshi et al. 1994). However, a functional role of the cleaved amelogenins studied here in enamel mineral formation cannot be ruled out, since these molecules may significantly contribute to the organization of the enamel matrix *in vivo*, as may other amelogenin degradation and alternative splice products (e.g., Lau et al. 1992), as well as small proportions of non-amelogenin proteins found in the developing enamel matrix. The present data clearly indicate that rM166 and P148 self-assemble to form large assemblies, as indicated by marked increased in light scattering (Fig. 3), with loose association between nanospheres under physiological pH conditions (Fig. 5b). The formation and nature of such assemblies are again highly sensitive to pH and might also be subject to cellular (ameloblast) pH regulation during amelogenesis.

More specifically, amelogenin self-assembly may be affected by ions or through hydrophobic/hydrophilic interactions with other macromolecules present in the extracellular milieu during enamel formation. Previously, it was shown that enamel matrix extracts from bovine (Kuboki et al. 1998; Nikiforuk and Simmons 1965) and porcine (Wen et al. 1999) developing teeth, containing a mixture of proteins, form weak, reversible gels. The formation and properties of such gels have been shown to be affected by the presence of mineral ions (Kuboki et al. 1998) and temperature (Nikiforuk and Simmons 1965). In particular, it has been shown (Nikiforuk and Simmons 1965; Wen et al. 1999) that amelogenin extracts form clear gels at 4°C, and that these gels transform into opaque masses when the temperature is increased above 18°C. In the present study, based on our DLS data (Figs. 2, 3), gel formation was not observed at 4°C. Several factors may have contributed to these different observations. First, a lower protein concentration was used in the present study (2mg/mL) in comparison to 7.3 mg/mL (Nikiforuk and Simmons 1965) or even higher concentrations (Wen et al. 1999) used in these referenced studies. Second, all experiments in the present study were performed with pure proteins, that is, either the full-length molecule or one specific cleavage product, as opposed to enamel extracts containing several different proteins and various amelogenin cleavage products. Although systematic studies like those carried out here using purified proteins allows one to test specific hypotheses to enhance our understanding of factors that influence enamel protein assembly, a potential limitation is that such experimental conditions do not reflect the

more complex *in vivo* environment, containing a pool of amelogenin cleavage and alternative splice products, as well as other molecules and mineral ions. Hence, additional studies using mixtures of specific proteins and additives are warranted.

Furthermore, *in vitro* studies with synthetic organic molecules have shown a critical interplay between crystal growth and organic matrix assembly (Colfen and Mann 2003), demonstrating that a subsequent cooperative reorganization of hybrid inorganic-organic nanostructures can give rise to the formation of hierarchical structures. These findings have a direct bearing on the mechanism of dental enamel formation and the present investigation, as was recently demonstrated *in vitro* using recombinant amelogenins (Fowler et al. 2006; Beniash et al. 2005). In particular, we have shown (Beniash et al. 2005) that parallel arrays of apatitic crystals, resembling forming enamel rods, formed in the presence of rM179 only when amelogenin assembly and mineralization were induced simultaneously, whereas rM166 had no effect on crystal organization. These findings clearly suggest that growing enamel crystals can also influence the organization of the full-length amelogenin assembly that ultimately serves to guide the formation of crystal arrays similar to those observed in developing enamel. Consistent with this idea, similar chains of nanospheres have also been observed in TEM studies of both dehydrated resin-embedded (Fincham et al. 1995) and non-dehydrated freeze-fractured sections of forming dental enamel (Robinson et al. 1981). These collective observations, along with the results presented here, provide evidence to support the notion that the full-length amelogenin can uniquely assemble *in vivo* into higher-order structures, similar to those observed in the present study. Conceivably, such structures could serve to guide the formation of organized arrays of enamel crystals as seen in the secretory stage of enamel formation. As noted in the Introduction, an interaction of specific domains of amelogenin to the retreating ameloblast may also serve to align amelogenin nanospheres and help guide enamel mineral growth *in vivo* (Paine et al. 2000).

In conclusion, the results of the present study strongly support the hypothesis that the self-assembly and formation of higher order structures of amelogenins *in vivo* is primarily regulated by pH. Furthermore, our data indicate that at a given pH the full-length amelogenins assemble differently compared to amelogenin cleavage products that lack the hydrophilic C-terminus. These results strongly suggest that the tightly controlled changes in pH observed during secretory amelogenesis may serve to regulate the functions of the full-length amelogenin and possibly that of various amelogenin cleavage products.

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rM179* - PLPPHPGSPGYINFSYEVL TPLK WYQSMIR
rP172 - PLPPHPGHPGYINFSYEVL TPLK WYQN MIR

QPYPSYGYEPMGGWLHHQIIPVLSQQHPPSH
 HPYTSYGYEPMGGWLHHQIIPVVSQQTPQSH

TLQPHHHLPVVPAQQPVAPQQPMMPVPGHHS
 ALQPHHHIPMVPAQQPGIPQQPMMLPGQHS

MTPTQH HQPNIPPSAQQPFQ-QPFQPQAIPP
 MTPTQH HQPNLPLAQQPFQ PQVPQPH--

QSHQPMQPQSPLHPMQPLAPQPPLPLFSMQ
 --- QPLQPQSPMHPIQPLLQPPLPPMFMSMQ

PLSPILPELPLEAWPATDKTKREEVD**
 SL --LPDLPLEAWPATDKTKREEVD**

* lacks N-terminal methionine and PO₄
 ** hydrophilic at pH7.4 - WPATDKTKREEVD

-+ ++ -- -

rM166 - same as rM179, but lacking the hydrophilic C-terminus
P148 - same as rP172, but with N-terminal methionine and PO₄
 (S-16); lacking 25 C-terminal amino acids

Alignment from Hu et al. 1996

Figure 1.
Aligned amino acid sequences of recombinant and native mouse and pig amelogenins used in the present study.

Figure 2.

DLS: R_H of rP172 (2mg/mL) in TRIS/HCl buffers *versus* temperature: **a.** buffer III was adjusted to pH 7.2 at 37°C (—□—); **b.** buffer II was adjusted to pH 7.2 at 25°C (- -Δ- -); and **c.** buffer I was adjusted to have pH 7.2 at 13°C (—○—). Note that pH rather than temperature triggers the formation of large aggregates, indicated here by a sharp increase in R_H (hundreds of nanometers) causing the scattering intensities to increase beyond the detector limits. The large standard deviations in R_H values observed at 25°C and at 33°C - 37°C are due to the presence of two different particle populations, indicating a rearrangement of initially formed nanosphere protein assemblies (~13 nm) prior to the formation of larger assemblies

Figure 3.

DLS data showing similar particle size (~13 nm) for both full-length and cleaved amelogenins at pH values above 7.8. The buffer pH was adjusted to pH 7.20 at 37 °C. The full-length amelogenins rM179 and rP172 subsequently form higher order assemblies (indicated by a sharp rise in R_H) at pH ~7.2, whereas the cleaved proteins do so at significantly higher pH values: P148 (pH 7.7), rM166 (pH 7.5). Standard deviations in these measurements are not shown.

Figure 4.

Turbidity measurements at 595nm. Samples in TRIS/HCl buffer, adjusted to pH 7.2 at 37°C, were prepared on ice and transferred into a temperature controlled sample holder, kept at a constant temperature of 37°C. A marked increase in turbidity (OD) was observed for all samples, followed by a slower decrease with time. See text for further discussion.

Figure 5.

a) P148 (2mg/mL) at pH 7.2 after 20 min: spherical particles (11 ± 2.5 nm in diameter) were found that were similar in size to those observed with the full-length amelogenin rP172 (Fig. 6a). Nearly identical results were obtained for the cleaved mouse amelogenin, rM166.

b) P148 (2mg/mL) at pH 7.2 after 150 min: spherical particles were observed that are bigger (20 ± 4.4 nm in diameter) than those observed at 20 min and far more abundant than those observed with the full-length amelogenin, rP172 (Fig. 6b). No elongated structures made up of connected spheres were observed, in contrast to that seen in rP172 samples (Fig. 6b). Similar spherical structures were observed for the cleaved mouse amelogenin, rM166.

Figure 6.

a) rP172 (2mg/mL) at pH 7.2 after 20 min: predominantly spherical particles (11 ± 2.6 nm in diameter) were observed, along with some chain-like structures that consisted of apparently tightly connected smaller spheres (9 ± 2.8 nm in diameter). Nearly identical results were obtained for the full-length mouse amelogenin, rM179.

b) rP172 (2mg/mL) at pH 7.2 after 150 min: tightly connected elongated assemblies were found to be predominant ($L/W = 7.8 \pm 4.8$ nm). However, compared to P148 and rM166, less material adhered to the grid, presumably as a result of more extensive separation of the protein assembly from solution. Nearly identical results were obtained for the full-length mouse amelogenin, rM179.

Table 1

Mean hydrodynamic radii (R_H) of full-length and cleaved mouse and pig amelogenins under acidic conditions (pH <4) from 4 to 43°C determined by DLS and calculated radii for individual molecules^c.

<i>Protein</i>	<i>rP172</i>	<i>P148</i>	<i>rM179</i>	<i>rM166</i>
Isoelectric Point pI ^a	7.05	7.94	7.05	7.23
Measured R_H [nm] in DDW ^b	1.1 ± 0.04	1.01 ± 0.07	1.4 ± 0.05	1.16 ± 0.06
Measured R_H [nm] in MOPS ^c	1.6 ± 0.13	1.2 ± 0.15	1.0 ± 0.06	1.0 ± 0.13
Calculated Radius [nm] ^d	1.78	1.69	1.80	1.75

^a SCRIPPS protein calculator v3.3 (<http://www.scripps.edu/~cdputnam/protcalc.html>)

^b pH 3.5; 2 mg/mL (n=1)

^c pH 3.7; 2 mg/mL, 80 mM MOPS buffer not pH adjusted (n = 1)

^d Based on mean partial specific volumes of soluble globular proteins (Harpaz et al. 1994).

pH values of control (no protein) and protein solutions (2mg/mL) prepared in TRIS/HCl buffers adjusted to pH 8.0 at 25°C, and to pH 7.2 at 25°C and 37°C, measured at different temperatures^d.

Table 2

Measured at Temp [°C]	Control		Sample		Control		Sample		Control		Sample	
	TRIS/HCl pH 8.0 adj. 25 °C		rP172 in TRIS/HCl		TRIS/HCl pH 7.2 adj. 37 °C		rP172 in TRIS/HCl		TRIS/HCl pH 7.2 adj. 25 °C		rM179 in TRIS/HCl	
on ice	8.79		8.79		8.13		8.32		7.98		7.92	
25	8.04		8.04		7.46		7.40		7.24		7.20	
37	7.76		7.74		7.20		7.15		6.96		6.86	
43	7.60		7.60		7.06		7.00		6.85		6.72	

^d Small discrepancies between control and sample pH (0.1 – 0.13 pH units) were observed when pH values of the TRIS/HCl buffers approached or went below the lower pH limit (i.e., ~ pH 7.1) of its buffer capacity.

Table 3
TEM measurements of amelogenin samples at pH 7.2 incubated at 37°C for 20 and 150 minutes* .

Protein	Shape and Dimension			Difference 20 – 150 min	
	20 minutes	N	150 minutes	N	p-value
P148 rP172	Spheres: D: 11 ± 2.5 nm	150	Spheres: D: 20 ± 4.4 nm	150	< 0.001
	Spheres: D: 11 ± 2.6 nm	150	Chains: W: 7 ± 2.1 nm L: 51 ± 26.2 nm L/W: 7.8 ± 4.8	73	< 0.001
rM179	Spheres: D: 10 ± 2.5 nm	73	Chains: W: 7 ± 1.3 nm L: 58 ± 27.0 nm L/W: 9.2 ± 5.1	97	< 0.001
rM166	Spheres: D: 11 ± 2.4 nm	150	Spheres: D: 9 ± 2.5 nm	150	< 0.001

* From 4 to 5 different micrographs, where N is the number of particles measured; D: diameter, W: width, L: length; L/W: aspect ratio.