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In vitro study on the interaction between the 32 kDa enamelin and amelogenin

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Abstract

Enamel extracellular matrix components play vital roles in controlling crystal nucleation and growth during enamel formation. We investigated the interaction between the 32 kDa enamelin fragment and amelogenin using immunochemical and biophysical methods. Immunoprecipitation studies revealed that the 32 kDa enamelin and amelogenin eluted together from a Protein A column. Dynamic light scattering results showed that the 32 kDa enamelin had a profound effect on amelogenin assembly at pH 8.0, causing partial dissociation of the nanospheres, in a dose-dependent manner. The appearance of an isodichroic point and the shifting and intensity decrease of the ellipticity minima in the circular dichroism spectra of amelogenin following the addition of the 32 kDa enamelin were indicative of conformational changes in amelogenin and of a direct interaction between the two macromolecules. Our results collectively demonstrate that the 32 kDa enamelin has a direct interaction with amelogenin in vitro. Our current studies provide novel insights into understanding possible cooperation between enamelin and amelogenin in macromolecular self-assembly and in controlling enamel mineral formation.

Keywords

Enamel; 32 kDa enamelin; Amelogenin self-assembly; Immunoprecipitation; Circular dichroism; Dynamic light scattering

1. Introduction

The components of enamel extracellular protein matrix are critical for the formation of well-organized carbonated hydroxyapatite mineral. The matrix is composed of the dominant amelogenin (about 90%); two proteinases, matrix metalloproteinase 20 (MMP-20) and kallikrein 4 (KLK4); and two glycoproteins, enamelin and ameloblastin (Robinson et al., 1995; Moradian-Oldak and Paine, 2008). During enamel formation, ameloblasts synthesize and secrete enamel proteins into the extracellular space and these proteins are subject to a series of proteolytic cleavages. A number of in vitro and in vivo studies have demonstrated that extracellular matrix proteins play key roles in controlling the nucleation, growth, and organization of hydroxyapatite crystals during enamel formation (Gibson et al., 2001; Paine et al., 2001; Iijima and Moradian-Oldak, 2004; Habelitz et al., 2004; Beniash et al., 2005).

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Since it is the most abundant protein in the matrix, the importance of amelogenin supramolecular assembly in enamel mineralization has been extensively studied. It has been reported that amelogenins self-assemble to form nanospheres that further assemble to form higher order structures in vitro (Margolis et al., 2006; Moradian-Oldak et al., 1998, 2000, 2006). Despite recent advances in the identification of other enamel proteins that are critically required for normal enamel formation, knowledge of the function of enamelin is still limited, and the mechanism of crystal nucleation and the interactions among enamel proteins remain unclear.

Porcine enamelin, a phosphorylated glycoprotein, is secreted as a 186 kDa precursor. Among a series of cleavage products, the 32 kDa enamelin is the most stable fragment, extending from Leu¹⁷⁴ to Arg²⁷⁹ of the 186 kDa enamelin. It is hydrophilic and acidic with two phosphorylated serines and three glycosylated asparagines (Hu and Yamakoshi, 2003). Limited structural and functional characteristics for the 32 kDa enamelin have been reported previously (Fukae et al., 1996; Dohi et al., 1998; Yamakoshi, 1995). The secondary structure of the 32 kDa enamelin has a high content of α -helix conformation and undergoes a conformational change as a function of calcium ion concentration (Fan et al., 2008). Studies also showed that the 32 kDa enamelin binds to apatite crystals with high affinity (Tanabe et al., 1990), promotes nucleation of apatite crystals (Bouropoulos and Moradian-Oldak, 2004), and induces elongation of apatite crystals grown in agarose gel (Hu et al., 2008). These in vitro studies highlight the important functions of enamelin in controlling crystal nucleation or growth. Direct evidence for the notion that enamelin is absolutely essential for proper enamel mineralization has been obtained from animal models (Hu et al., 1997, 1998, 2001, 2008; Pavli et al., 2007). A recent study of enamelin knockout/LacZ knockin mice showed that animals with the *ENAM*^{+/-} genotype presented discolored and rapidly worn mandibular incisors, and that *ENAM*^{-/-} mice had no true enamel. Mutations in *ENAM* also cause *amelogenesis imperfecta* (AI), a hereditary disease of enamel malformation (Hu et al., 2008). Among these *ENAM* mutations, some occur in the regions associated with the sequence of the 32 kDa enamelin, highlighting the importance of this segment in controlling normal enamel formation (Masuya et al., 2005). Despite the recent efforts and advances in understanding of the structure and function of enamelin (Hu et al., 1997, 1998, 2000, 2001, 2008), its role in the enamel matrix and possible interactions with other enamel proteins are still largely unexplored. In particular, the molecular mechanisms of enamelin action still remain to be elucidated.

To better understand the role and mechanism of action of enamelin protein during enamel biomineralization, we investigated the interaction between the 32 kDa enamelin and the dominant enamel protein, amelogenin, using both biochemical and biophysical techniques. Based on previous immunohistochemistry results showing a prominent presence of 32 kDa enamelin in the inner layer of crystallite-containing rod and interrod areas of the enamel matrix (Uchida et al., 1991), we first applied immunoprecipitation using enamel matrix extract and a mixture of recombinant amelogenin with purified 32 kDa enamelin to study protein-protein interaction between these two enamel matrix proteins. In addition, dynamic light scattering (DLS) and circular dichroism (CD) were employed to analyze the interaction between a recombinant amelogenin (rP172) and purified 32 kDa enamelin in vitro.

2. Materials and methods

2.1. Extraction, purification and characterization of the 32 kDa enamel

The 32 kDa enamel was extracted following the method described previously (Yamakoshi, 1995). In brief, pooled enamel samples scraped from second and third unerupted molars of freshly dissected six-month-old pig jaws (Farmers John Clougherty Co., Los Angeles, CA, USA through Sierra For Medical Sciences, Santa Fe Springs, CA, USA) were homogenized in 50 mM Sørensen buffer (pH 7.4) in the presence of proteinase and phosphatase inhibitors. This extraction process was repeated three times. The combined supernatant was processed and purified by reverse-phase high performance liquid chromatography (RP-HPLC) as reported previously (Fan et al., 2008). The purified 32 kDa enamel was characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), stains-all staining, and Edman degradation (Fan et al., 2008).

2.2. Expression and purification of recombinant amelogenins (rP172 and rP148)

Two recombinant porcine amelogenins, rP172 or rP148 were expressed in *Escherichia coli*, purified using RP-HPLC, and characterized as previously described (Ryu et al., 1999; Sun et al., 2006). The rP172 polypeptide is analogous to the full-length porcine amelogenin P173, lacking only the first methionine and a phosphate on Ser¹⁶ (Yamakoshi et al., 1994; Fincham and Moradian-Oldak, 1993), while rP148 is analogous to the major amelogenin proteolytic product (P148 or the “20k”) lacking the 24 C-terminal amino acids the from the full-length amelogenin (amino acids 2–149) (Sun et al., 2006).

2.3. Preparation of proteins for immunoprecipitation

Two enamel protein samples, one an in vivo extract and the other an in vitro mixture, were used to examine the enamel–amelogenin interaction. The native enamel protein extract used for the immunoprecipitation study was prepared from third molars using PBS buffer (20 mM, pH 7.4) extraction and concentrated by lyophilization. The in vitro mixture of rP148 (0.22 mg/ml, 48 µl) and purified 32 kDa enamel (0.17 mg/ml, 2 µl) was incubated in 20 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.4, 0.15 M NaCl, 2.0 mM CaCl₂) at room temperature for 3 h before applying it to the Protein A column.

2.4. Immunoprecipitation

The immunoprecipitation was carried out using an Immuno-Pure (Protein A) IgG purification kit (Pierce, Rockford, IL, USA) containing washing and elution buffers. In brief, after equilibrating the Protein A column with 5 ml of binding buffer, 10 µl recombinant mouse amelogenin antibody solution (5.0 µM) was loaded onto the column. Diluted sample (50 µl, native extract or mixture of rP148 with enamel) was applied into the column and allowed to flow completely into the gel. The Protein A column was washed with 15 ml of binding buffer. The bound proteins were eluted by 5 ml of IgG elution buffer and the eluted solution was collected in fractions. The fractions were neutralized by adding binding buffer, and lyophilized for western blot detection.

2.5. Western detection of proteins

Detailed methods for amelogenin and enamel detection were applied as published (Hu et al., 2008). In brief, the proteins in the samples and controls were resolved using 10% SDS–PAGE. The gel was electrotransferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA), which was then blocked extensively and washed thoroughly with Tween–Tris Buffered Saline (TTBS) buffer (Bio-Rad, Hercules, CA, USA). Amelogenin epitopes were detected using a rabbit polyclonal antibody raised against recombinant mouse amelogenin rM179 at a dilution of 1:2000, while enamel was detected using affinity purified rabbit antibodies (3.2 μ M, 1:1000) raised against the enamel-derived peptide EQDFEKPKEKDPPK (gift from Dr. Jan Hu, University of Michigan). After washing with TTBS, the blots were incubated with anti-rabbit IgG secondary antibody (1:3000) conjugated to horse-radish peroxidase (Bio-Rad, Hercules, CA, USA). Both amelogenin and enamel were detected by enhanced chemiluminescence (ECL, GE Healthcare Biosciences).

2.6. Dynamic light scattering (DLS)

The interaction between the 32 kDa enamel and recombinant amelogenin (rP172, 0.18 mg/ml) in 20 mM Tris (2.0 mM CaCl_2 , 0.15 M NaCl) was investigated by DLS at pH 5.8 and pH 8.0. The rP172 solution was first measured and then enamel solution (5.3 μ M in 20 mM Tris with 0.15 M NaCl (pH 7.4)) was added at various ratios. Each mixture was incubated for 30 min at room temperature. DLS measurements were performed on a DynaPro-99EMS/X instrument equipped with a solid-state laser operating at 655 nm at a fixed angle of 90° with a temperature controlled MicroSampler at 20 °C (Wyatt Technologies, Santa Barbara, USA), as previously described (Du et al., 2005). A data collecting strategy of multiple runs at short acquisition time was employed. The acquisition time for each run was set at 10 s. The experiment continued for 50–60 runs, which corresponded to 8–10 min. The data analysis software (Dynamics V6.3.01) was able to analyze the correlation function for each individual run as well as for the whole experiment. The SOS (sum of squares error for a Cumulants fit), amplitude, and baseline were within acceptable limits for the collected data, and the channel cut off was set at 160. The autocorrelation function of the signal from the scattered intensity was measured by a multi-channel digital correlator. The normalized intensity correlation function was analyzed by a regularization method, included in the data analysis software package, to give the information on the distribution of the exponential decay function with decay rate Γ . The translational diffusion coefficient was determined through $\Gamma = Dq^2$ where q is the scattering vector. The hydrodynamic radius R_H was calculated from D via the Stokes–Einstein equation, $R_H = kT/(6\pi\eta D)$, where k is the Boltzmann constant and η the solvent viscosity at temperature T . The mass distribution of the radius was calculated from the intensity distribution via a simplified form of the Rayleigh equation. The molecular weight of molecules was estimated from DLS data using a calibration curve developed from protein standards of known molecular weight and size. All data for the enamel–amelogenin mixture were modeled as Rayleigh spheres using a Cumulants analysis.

2.7. Circular dichroism (CD) spectroscopy

CD measurements for amelogenin and its interaction with the 32 kDa enamel were conducted on a JASCO J-810 spectropolarimeter calibrated using a 0.06% (+)-10-camphorsulfonic acid solution. Instrument optics and sample chamber were continually flushed with 20 L/min of dry N₂ gas. The recombinant amelogenin (rP172) was dissolved in 20 mM Tris buffer (2.0 mM CaCl₂, 0.15 M NaCl) to get solutions of 0.18 mg/ml at pH 5.8 and 0.11 mg/ml at pH 8.0. The purified 32 kDa enamel fragment was dissolved in 20 mM Tris buffer (pH 7.4, Ionic Strength (IS) = 0.15) to obtain a protein concentration of 0.17 mg/ml (5.3 μM). The CD spectra were measured at room temperature in a 1 mm path-length quartz cell (300 μl) by using a scanning speed of 50 nm/min, a time response of 1 s, a bandwidth of 1 nm, and average of eight scans. The CD spectra for the interactions between enamel and rP172 were taken after a 30-min incubation at room temperature. CD spectra were expressed as the mean residue ellipticity, $[\theta]_{\text{mrw}}$ (deg cm² dmol⁻¹). $[\theta]_{\text{mrw}}$ was calculated using the equation,

$$[\theta]_{\text{mrw}} = [\theta]_{\lambda} \times \text{mrw} / 10 \times l \times c$$

where $[\theta]_{\lambda}$ is the observed ellipticity, mrw is the mean residue weight, 'l' is the optical path-length, and 'c' is the concentration of the protein (mg/ml). The mean residue weight is defined as $M/N - 1$ where M is the molecular mass ($M_{\text{rP172}} = 19572.5$; $M_{\text{enamelin}} = 32000$ Da) and N is the number of amino-acid residues.

3. Results

3.1. Immunoprecipitation of native and recombinant proteins

To analyze the association between amelogenin and enamel, a polyclonal antibody to rM179 specific to amelogenin proteins was used to immunoprecipitate amelogenin and its associated proteins from two samples: an in vitro mixture of rP148 with purified 32 kDa enamel and an in vivo native enamel protein extract. Based on immunohistochemistry showing a prominent presence of the 32 kDa enamel in the inner layer of the enamel matrix rich with the 20 kDa amelogenin, the recombinant amelogenin rP148 was used for the immunoprecipitation study. The immunoprecipitation results from western blots against rM179 and rabbit antibody raised against the enamel peptide are presented in Fig. 1. The western blot results for the precipitated and eluted material derived from the in vitro mixture of rP148 with 32 kDa enamel presented a strong band of 18 kDa (rP148) (lane 3, Fig. 1A) and a distinct band of 32 kDa enamel (lane 3, Fig. 1B). In comparison, the precipitation from the in vivo enamel protein extract had two major bands corresponding to 25 kDa and 23 kDa amelogenins, a weak band corresponding to 13 kDa amelogenin (lane 2, Fig. 1A), and a 32 kDa band corresponding to enamel fragment (lane 2, Fig. 1B). The co-immunoprecipitation results therefore demonstrated that the rM179 antibody precipitated amelogenin together with the 32 kDa enamel from both the in vitro sample and the in vivo extract.

The elution fractions from the Protein A column were also characterized by SDS-PAGE with silver staining. For the eluted material derived from the in vivo extract, there were

several bands present in the sample: four major bands representing amelogenin with molecular weights ranging from 11 to 25 kDa and a weak high molecular weight band representing the 32 kDa enamelin (lane 2, Fig. 2A). For the material eluted from the mixture of rP148 and purified enamelin, there are two clear bands, a strong one of 18 kDa for rP148 amelogenin and a high molecular weight band for the 32 kDa enamelin (lane 2, Fig. 2B). The silver staining thus also manifested the co-precipitation of enamelin and amelogenin, suggesting an intimate association between the dominant amelogenin and enamelin isolated from the enamel matrix. In a control experiment without loading rM179 antibody, neither amelogenin nor enamelin was bound to the Protein A column following the wash with binding buffer (data not shown).

3.2. Biophysical studies on amelogenin and enamelin interaction

On the basis of the immunoprecipitation results showing the close association between amelogenin and the 32 kDa enamelin, we employed biophysical methods, DLS and CD, to further study the interaction between, and co-assembly of, these two proteins in solution. Due to the highly aggregated nature of rP148 under neutral or weakly basic conditions (Moradian-Oldak et al., 2002), we chose rP172 instead of rP148 in order to optimize the amelogenin solubility for the biophysical studies. Early studies also showed a relative poor solubility of rP172 at pH 7.4 when compared with acidic and basic conditions (Moradian-Oldak et al., 1994). For these reasons, we investigated the interactions between the 32 kDa enamelin and amelogenin (rP172) using DLS and CD at pH 5.8 and 8.0 in 20 mM Tris buffer, instead of at pH 7.4, despite the fact that neutrality better represents physiological conditions.

DLS analyses provided the estimated hydrodynamic radii (R_H), molecular weight (MW), and mass distribution of particles in the rP172–enamelin solutions in 20 mM Tris buffer (pH 5.8 and 8.0) (Table 1). The results demonstrate that the influence of the 32 kDa enamelin on the rP172 particle size is substantially different in acidic and basic conditions. At pH 5.8, rP172 amelogenin has a monodisperse size distribution with a mean R_H of 2.9 nm and an estimated MW of 41 kDa, suggesting dimers of rP172 molecules (Moradian-Oldak et al., 1994). When the 32 kDa enamelin was added to rP172 in molar ratios of 1:100 and 1:50, the particle size distribution in the rP172–enamelin solution remained monodisperse and the size was not affected at this pH. This is possibly due to the fact that there are still amelogenin-dominated particles in the mixture. At a 1:10 ratio of enamelin to rP172, the size distribution of particles in the mixture is still monodisperse at pH 5.8 but with an increase of R_H to 3.8 nm. The estimated MW for the R_H 3.8 nm particles is 77 kDa, which is likely due to the formation of a complex between two rP172 molecules (based on MW 20 kDa for rP172) and one 32 kDa enamelin, indicating a direct interaction between these two enamel proteins.

The influence of the 32 kDa enamelin on the self-assembly behavior of rP172 at pH 8.0 was remarkable. Without enamelin, the self-assembled rP172 nanospheres exhibited a bimodal size distribution; one major population had a mean R_H of 17.6 nm while another minor population had an average R_H of 62.9 nm (Table 1). The minor population may well be the result of further assembly of amelogenin nanospheres (Moradian-Oldak et al., 1994, 1998, 2006; Du et al., 2005). The nanosphere in the major group (R_H of 17.6) had a mean MW of

2759 kDa, which is consistent with the results previously reported (Margolis et al., 2006; Moradian-Oldak et al., 1994, 1998; Wiedemann-Bidlack et al., 2007). However, the estimated R_H and MW of the nanospheres in the rP172–enamelin solution at pH 8.0 become smaller upon addition of the 32 kDa enamelin. When enamelin was initially added to rP172 at a molar ratio of 1:100, the average R_H of nanospheres in the major and minor populations decreased to 15.1 nm and 54.1 nm, respectively. At a 1:50 ratio of enamelin to rP172, the average R_H of the nanospheres from the major and the minor populations was reduced to 11.8 nm and 49.3 nm, respectively. At a high ratio of enamelin to rP172 (1:10), the average R_H of the nanospheres decreased further to 4.3 nm in the major group and 32.7 nm in the minor population. The nanospheres detected by DLS at the 1:10 ratio might be either enamelin–amelogenin complexes or smaller rP172 nanospheres. We interpret these data to suggest that the particles with a mean MW of 104 kDa are likely formed by four rP172 molecules and one 32 kDa enamelin, generating an enamelin–amelogenin complex, or by five rP172 molecules, giving rise to a highly disassembled amelogenin particle. Although it is not possible to unambiguously define the composition of these particles, the DLS data clearly demonstrate partial dissociation of amelogenin nanospheres in a dose-dependent manner with addition of 32 kDa enamelin, showing a direct interaction and co-assembly of these two enamel proteins.

In a control test, in which the same volume of the 32 kDa enamelin was added to a buffer solution without rP172, DLS measurement using either a globular or a linear model could not detect enamelin particles at concentrations equivalent to the mixtures with ratios of 1:100 and 1:50 of enamelin to rP172 (Table 2). We suggest that there is no aggregation at these very low concentrations for the 32 kDa enamelin. At a higher concentration of 1.0 μM (equivalent to that in the 1:10 ratio mixture), the particle size of the main population of enamelin is over 4100 nm, which is significantly different from the particle size we observed in the rP172–enamelin solution. This is because the 32 kDa enamelin has a strong self-binding nature and forms large aggregates when it reaches a critical concentration (Paine and Snead, 1997). On the other hand, DLS data also imply that when the 32 kDa enamelin and amelogenin molecules associate with each other, their interaction prevents large aggregations of enamelin from forming in the solution.

Additional evidence for the formation of enamelin–amelogenin complexes was obtained by analyzing the intensity of photons detected by DLS. We found that at pH 5.8, the intensity of photons increased following the addition of enamelin (Fig. 3A). However, at pH 8.0, the photon intensities decreased initially after enamelin was added (molar ratios of 1:100 and 1:50) indicating a decrease in nanosphere size. When the enamelin to rP172 ratio was increased to 1:10 at pH 8.0, the photon intensity increased again, suggesting that the scattering number of nanospheres may play a major role at a relatively high enamelin to rP172 ratio (Fig. 3B). The enamelin–rP172 mixture became polydispersed and some large nanospheres did occur when enamelin was added to rP172 at the ratio 1:10.

The CD spectrum of rP172 amelogenin has a strong negative ellipticity around 201–203 nm, which is characteristic of an unordered polyproline type II structure (Lakshminarayanan et al., 2007). At pH 5.8, the minimum $[\theta]_{\text{mrw}}$ of rP172 at 203 nm is $-2.7 \times 10^3 \text{ deg cm}^2 \text{ dmol}^{-1}$ (Fig. 4A) and it is $-1.5 \times 10^3 \text{ deg cm}^2 \text{ dmol}^{-1}$ at 203 nm at pH 8.0 (Fig. 4B). When the 32

kDa enamel was added to rP172 at a molar ratio of 1:100, presumably resembling to the ratio of these two proteins in vivo, the minimum decreased at both pH's. With increasing ratios of enamel to rP172 (1:50 and 1:10), the intensity of the minima decreased further with a steeper slope for the change in pH 8.0 solution, as shown in the insets of Fig. 4. This implies that there may be a relatively higher degree of influence of enamel on amelogenin under basic pH conditions, which is consistent with the DLS measurements. At the same time, an isodichroic point appeared at 194 nm in pH 5.8 and at 195 nm in pH 8.0 solution, which strongly supports the conformational changes of amelogenin as a result of an influence of the 32 kDa enamel (Sanchez et al., 1997; Chowdary et al., 2007). These conformational changes, as detected by CD spectra, further support the notion that the 32 kDa enamel molecules co-assemble with amelogenin.

4. Discussion

Macromolecular assembly and protein–protein interactions are believed to play important roles in the process of matrix-mediated enamel biomineralization (Fincham et al., 1999; Margolis et al., 2006). Our studies explored the role of enamel protein in macromolecular assembly during enamel biomineralization. We applied three independent techniques to demonstrate that an in vitro interaction between the 32 kDa enamel and the dominant enamel protein amelogenin does take place.

In a recently described case of a hypoplastic AI with *ENAM* gene mutations, alterations in codon 197 resulted in the loss of Ser²¹⁶ and of all three N-glycosylation sites on the 32 kDa enamel polypeptide (Pavli et al., 2007). Such defects could have a direct impact on amelogenin–enamel interactions.

Our present data support a hypothesis that the 32 kDa enamel, through direct interactions with amelogenin and in a cooperative manner, may regulate amelogenin assembly, which in turn controls the nucleation and the oriented and elongated growth of enamel crystals. Such a hypothesis is plausible because both proteins appear to be present in the inner layer of developing enamel and are co-localized within that layer (Uchida et al., 1991). Our in vitro observations indicate a close association of the 32 kDa enamel with amelogenin, in good agreement with the results of early immunohistochemical studies (Uchida et al., 1991).

The 32 kDa enamel has the potential to interact with both the full-length (P173) and the truncated amelogenins lacking the C-terminal region (P161 or P148) through the tyrosyl motif at the N-terminal region that is common to all these forms of amelogenin (Ravindranath et al., 1999). Considering the fact that the in vivo enamel matrix consists of the dominant amelogenin and low abundance enamel, ameloblastin and enzymes, we envisage these proteins cooperating synchronically with one another in the enamel formation process. The immunoprecipitation experiments present direct evidence that amelogenin and enamel were co-precipitated from Protein A column using the rM179 antibody which specifically recognizes amelogenin proteins. The co-immunoprecipitation of amelogenin and enamel is likely due to co-assembly of these two proteins so that enamel was held in the column through binding to amelogenin which in turn was bound to rM179 antibody. In both the in vivo extract and the in vitro mixture samples, the 32 kDa enamel was

immunoprecipitated with amelogenins, most likely due to the glycosylated sites of enamel binding to the tyrosyl rich N-terminal region of amelogenin (Ravindranath et al., 1999). This observation is clear evidence that a direct interaction between the 32 kDa enamel and amelogenin leads to a complexation of these two enamel proteins, giving rise to co-precipitation of amelogenin and enamel.

Particle size distribution analysis of amelogenin–enamel mixtures by DLS indicated formation of complexes between the two proteins at acidic pH (5.8). Although the particles detected by DLS could be a mixture of rP172 dimers and enamel–amelogenin complexes at the ratio of 1:10 at pH 5.8, we suggest that the particles having R_H 3.8 nm are the result of enamel–amelogenin direct interactions. At pH 8.0, moreover, the 32 kDa enamel displayed a profound influence on the self-assembly property of amelogenin. Interestingly, upon sequential addition of the 32 kDa enamel at enamel–rP172 molar ratios of 1:100, 1:50, and 1:10, the particle size of the major population in the rP172–enamel solution decreased from initial 17.6 nm to 15.1 nm, 11.8 nm, and 4.3 nm. The decrease in average size of amelogenin-containing particles as a function of increasing enamel concentration is another indication that co-assembly between enamel and amelogenin molecules does take place. A schematic diagram representing the effect of enamel at different ratios on particle size distribution is shown in Fig. 5. It is assumed that hydrophobic amino-acid residues engage in Van der Waals interactions and amelogenin nanospheres would be in some form of equilibrium with individual amelogenin molecules (Aoba and Moreno, 1989; Fincham et al., 1998; Lakshminarayanan et al., 2007). Such an equilibrium of amelogenin self-assembly could be broken and shifted by the presence of proteins, ligands, or a change of pH or temperature. When the 32 kDa enamel was added to solution containing assembled amelogenin, the nanosphere size of rP172 in the solution became smaller, indicating a shifting of the amelogenin self-assembly equilibrium towards dissociation, in a dose-dependent manner. Based on earlier results, we speculate that when enamel binds to amelogenin through its sugar chains, its hydrophilic and acidic nature would stabilize the amelogenin molecules (possibly converting them to a more stable conformation) and thus inhibit the hydrophobic interactions among neighboring rP172 molecules. On the other hand, it is important to note that amelogenin also prevented the strong self-aggregation of enamel, indicating possible stabilization of enamel structure following its interaction with amelogenin.

Conformational changes of rP172 as a result of its co-assembly with the 32 kDa enamel were apparent in the CD experiments. The appearance of an isodichroic point accompanied by slight shifting and intensity decrease of minima in the CD spectra gave a solid indication of the conformational changes of rP172 when enamel was added. From the interpolated data of intensity change, a more steeply sloped curve at pH 8.0 suggested that the 32 kDa enamel has a higher degree of influence on the conformation of assembled amelogenin than on the amelogenin dimer in acidic condition. The above CD results were in line with the observations on particle size distribution using DLS analysis.

In conclusion, we have provided solid evidence for the interaction between amelogenin and the 32 kDa enamel by applying biochemical and biophysical assay. These results are contrary to the observations of two early studies, which both reported that enamel and

amelogenin failed to interact (Paine and Snead, 1997; Yamakoshi et al., 2003). The difference in observations may be due to the different testing methods applied and the limitations of these techniques. We suggest that the 32 kDa enamelin may play a profound role in regulating amelogenin supramolecular assembly, or in preventing unwanted aggregation of the proteolytic products in vivo. The precise nature of such interactions and the functions and relationships among other enamel matrix proteins are not known. Clearly, further studies are needed to fully understand the association and cooperation among enamel matrix proteins and the roles these proteins play during the mineralization process.

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Fig. 1. Immunoprecipitation of in vivo enamel protein extraction and mixture of rP148 and the 32 kDa enamelin fragment. (A) Western blot against rM179. (B) Western blot against enamelin antibody. Lane 1: control in vivo extraction, lane 2: in vivo extraction sample immunoprecipitated and eluted from Protein A column, lane 3: mixture of rP148 and enamelin sample immunoprecipitated and eluted from Protein A column.

Fig. 2.

Silver staining. (A) Lane 1: Std. Marker, lane 2: in vivo extract immunoprecipitated and eluted from Protein A column. (B) Lane 1: Std. Marker, lane 2: mixture of rP148 and the 32 kDa enamelin fragment immunoprecipitated and eluted from Protein A column.

Fig. 3.
The intensity of scattering of particles as determined by DLS in enamel-rp172 mixtures, at pH 5.8 (A) and pH 8.0 (B) Tris buffer.

Fig. 4.

CD spectra of rP172 interaction with the 32 kDa enamel fragment. (A) rP172 (0.18 mg/ml) in 20 mM Tris, 2.0 mM CaCl_2 , 0.15 M NaCl, pH 5.8; (B) rP172 (0.11 mg/ml) in 20 mM Tris, 2.0 mM CaCl_2 , 0.15 M NaCl, pH 8.0. (rP172 (—); enamel:rP172 = 1:100 (---); enamel:rP172 = 1:50 (.....); enamel:rP172 = 1:10 (-·-·-)). The insets show the minimum $[\theta]_{\text{mru}}$ change at different enamel ratios.

Fig. 5. Schematic diagram showing the effect of increasing concentration of enamel on the size distribution of amelogenin-containing particles. Numbers in parentheses represent the molar ratio of enamel to rP172.

Table 1

Hydrodynamic radii (R_H), molecular weight (MW), and mass distribution of particles in the enamelin-rP172 solutions (rP172, 0.18 mg/ml, 20 mM Tris buffer, 2.0 mM CaCl_2 , 0.15 M NaCl).

Enamelin:rP172	pH 5.8			pH 8.0		
	R_H (nm)	MW (kDa)	Mass (%)	R_H (nm)	MW (kDa)	Mass (%)
No enamelin	2.9 ± 0.34	41	99.4	17.6 ± 2.2	2759	91.0
				62.9 ± 13.1	54,494	9.0
1:100	2.9 ± 0.36	41	99.6	15.1 ± 1.7	1931	87.0
				54.1 ± 15.1	38,200	13.0
1:50	3.0 ± 0.44	43	99.9	11.8 ± 0	1089	87.8
				49.3 ± 12.4	30,723	11.4
				196.1 ± 17.1	778,545	0.9
1:10	3.8 ± 0.51	77	99.4	4.3 ± 0	104	96.9
				32.7 ± 11.3	11,770	3.0
				136.0 ± 18.2	330,282	0.2

Table 2

Hydrodynamic radii (R_H), molecular weight (MW), and mass distribution of particles of the 32 kDa enamelin at different concentrations in 20 mM Tris buffer (pH 7.4, 2.0 mM CaCl_2 , 0.15 M NaCl).

Concentration (μM)	R_H (nm)	MW (kDa)	Mass (%)
<i>Using globular model</i>			
0.1	0.1	0	100
0.2	0.2	0	100
1.0	52.8 ± 12	36,165	7.1
	189.9 ± 55	722,263	8.6
	4164.6 ± 1078	991×10^6	84.2
<i>Using linear model</i>			
0.1	0.2	0	100
0.2	0.1	0	100
1.0	111.8 ± 30	10,544	3.8
	747 ± 154	330,270	6.0
	4510 ± 771	86×10^6	90.2