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The 32 kDa Enamelin Undergoes Conformational Transitions upon Calcium Binding

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Abstract

The 32 kDa hydrophilic and acidic enamelins, the most stable cleavage fragment of the enamel specific glycoprotein, is believed to play vital roles in controlling crystal nucleation or growth during enamel biomineralization. Circular dichroism and Fourier transform infrared spectra demonstrate that the secondary structure of the 32 kDa enamelins has a high content of α -helix (81.5%). Quantitative analysis on the circular dichroism data revealed that the 32 kDa enamelins undergoes conformational changes with a structural preference to β -sheet as a function of calcium ions. We suggest that the increase of β -sheet conformation upon presence of Ca^{2+} may allow preferable interaction of the 32 kDa enamelins with apatite crystal surfaces during enamel biomineralization. The calcium association constant of the 32 kDa enamelins calculated from the fitting curve of ellipticity at 222 nm is $K_a = 1.55 (\pm 0.13) \times 10^3 \text{ M}^{-1}$, indicating a relatively low affinity. Our current biophysical studies on the 32 kDa enamelins structure provide novel insights towards understanding the enamelins-mineral interaction and subsequently the functions of enamelins during enamel formation.

Keywords

Enamelins; biomineralization; secondary structure; α -helix; β -sheet; circular dichroism; Fourier transform infrared

1. Introduction

Enamel biomineralization is an extracellular process that takes place in an enamel matrix constituting about 90% of amelogenin, with the remaining being proteinases, enamelins, ameloblastins, and amelotin (Moradian-Oldak et al., 2008; Robison et al., 1995; Margolis et al., 2006). Numerous in vitro and in vivo studies have demonstrated that extracellular matrix proteins play essential roles in controlling the nucleation, growth, and organization of hydroxyapatite crystals during enamel formation (Iijima and Moradian-Oldak, 2004; Beniash et al., 2005; Habelitz et al., 2004; Gibson et al., 2001; Paine et al., 2001). The significance 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 (Moradian-Oldak et al., 2006; Du et al. 2005). Although studies have supported the notion of enamel crystal orientation and habit being controlled

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through an organized assembly of the constituent proteins of the extracellular matrix, the mechanism of crystal nucleation and the function of other enamel proteins remain unclear.

Enamelin, a phosphorylated enamel specific glycoprotein that constitutes only a small percentage of the extracellular matrix (1-5%), plays a key role in enamel formation. The cDNAs for porcine (Hu et al., 1997), human (Hu et al., 2000) and mouse (Hu et al., 1998) enamelins have been recently characterized. The human gene for enamelins (*ENAM*) which is on the 4q21 chromosome contains 9 exons and is secreted as a 186 kDa precursor (Hu et al., 2000; Hu et al., 2001). Mutations in *ENAM* cause *amelogenesis imperfecta* (*AI*), a hereditary disease of enamel malformation (Pavlič et al., 2007). A recent homozygous (*Enam*^{-/-}) in *Enamelin*-null mice failed to form true enamel revealing that enamelins are critical for normal enamel formation (Hu et al., 2008). Once secreted, enamelins, like amelogenin, are subjected to a series of proteolytic cleavages. In developing porcine enamel, enamelins have been isolated with molecular weights of 25, 32, 45, 89, 142 and 155 kDa (Fukae et al., 1996). Among them, the 32 kDa enamelins are the most stable fragment of 186 kDa enamelins (extending from Leu¹⁷⁴ to Arg²⁷⁹) and its carbohydrate moieties have been characterized (Fukae et al., 1996; Dohi et al., 1998; Yamakoshi, 1995). The 32 kDa enamelins are hydrophilic and acidic with a pI 3.2, and are rich in proline (18.8%), glycine (12.3%), threonine (10.4%), and glutamic acid (9.4%) (Hu and Yamakoshi, 2003). It has two phosphorylated serines (Ser¹⁹¹ and Ser²¹⁶) and three glycosylated asparagines (Asn²⁴⁵, Asn²⁵² and Asn²⁶⁴) (Yamakoshi, 1995). Immunohistochemistry using antibody against the 32 kDa enamelins showed a prominent presence of the 32 kDa enamelins in the inner layer of crystallite-containing rod and interrod areas of the enamel matrix (Uchida et al., 1991). Furthermore, the 32 kDa enamelins were the only cleavage product that had high affinity to bind to apatite crystals (Tanabe et al., 1990), and caused elongation of the crystals grown in agarose gel (Hu et al., 2008) highlighting relevant functional properties of enamelins in controlling crystal nucleation or growth. The addition of 32 kDa enamelins onto amelogenins promoted the nucleation of apatite crystals (Bouropoulos and Moradian-Oldak, 2004). Despite the advances in understanding enamelins primary structure, the structural properties of enamelins and its interactions with ligands are still largely unexplored (Hu et al., 1997; 1998; 2000; 2001; Hu and Yamakoshi, 2003).

Here we applied circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy to study secondary structural preferences of the 32 kDa enamelins, isolated from developing porcine enamel, in the absence and presence of calcium ions. Elucidating structural features of the 32 kDa enamelins and the effect of calcium on its conformational transitions will contribute to the understanding of enamelins mineral interactions and therefore its function in enamel biomineralization.

2. Materials and methods

2.1 Materials

Sodium chloride, sodium phosphates monobasic and dibasic were purchased from Merck KGaA, Germany. Stains all was obtained from ICN Biochemicals Inc, Aurora, Ohio. Calcium chloride was purchased from Curtin Matheson Scientific Inc. Texas and was made a 40 mM stock solution for Ca²⁺-binding study with the 32 kDa enamelins. All other reagents were purchased from Sigma Chemical Co. Missouri and used as received.

2.2 Extraction and purification of the 32 kDa enamelins

The 32 kDa enamelins were extracted following the method described previously (Yamakoshi, 1995). In brief, the pooled enamel samples scraped from 2nd and 3rd 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) with proteinase and phosphatase inhibitors. This extraction process was repeated three times. The combined supernatant was treated with solid ammonium sulfate to make first a 40% saturation solution and then a 65% saturation. The resulting pellets were collected, which is rich in 32 kDa enamelins, re-suspended in 0.1% trifluoroacetic acid, and purified by reverse-phase high performance liquid chromatography (RP-HPLC), first using a C4 column (250×10 mm, Phenomenex) followed by a C18 column (250×10 mm, Phenomenex). Protein concentration was estimated by a Bio-Rad protein assay using bovine serum albumin as a standard following the method of Bradford (Bradford, 1976).

2.3 Characterization of the 32 kDa enamelins (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), stains-all staining and Edman degradation)

The SDS-PAGE was performed using 12% polyacrylamide precast slab gel (Invitrogen) containing 1% SDS according to the method reported previously (Laemmli, 1970). The samples were dissolved in loading buffers at a concentration of 0.2% w/v. The electrophoresis was carried out using a voltage of 120 V for 2 hours. The gel was stained with Coomassie Brilliant Blue solution and destained with 7.5% acetic acid and 5% methanol. The protocol for stains-all staining was adopted from that described previously (Campbell et al., 1983). In brief, after electrophoresis, the gel was rinsed three times with 25% (v/v) isopropanol followed by washing in 30-50 ml of the same solution on a shaker for 10 min. The rinsing and washing cycle was repeated three times. This ensures to removal of all SDS, which, if present, would cause the precipitation of stains-all dye. Isopropanol was then replaced by 30 ml of stains-all solution (20 mM Tris, 7.5% (v/v) formamide, 25% (v/v) isopropanol, followed by addition of 0.025% (w/v) stains-all). Due to the photosensitivity of stains-all, the gel was incubated in a light-tight container on an orbital shaker at room temperature for at least 2 hours. The stains-all showed a positive response with a blue band around 32 kDa, indicating that enamelins are stripped of Ca^{2+} after the RP-HPLC process (Campbell et al., 1983; Caday et al., 1986). For protein sequence analysis following SDS-PAGE, the gel was equilibrated in 3-[cyclohexylamino]-1-propanesulfonic acid buffer and was electrotransferred to polyvinylidene difluoride membrane (Millipore) at 50 volts for 30 minutes. Amino acid sequence analysis was performed at the Division of Biological Sciences Protein Sequencing Facility at UCSD.

2.4 Circular dichroism (CD) spectroscopy and calcium binding affinity determination

CD measurements for the 32 kDa enamelins and its interactions with Ca^{2+} were conducted on a JASCO J-810 spectropolarimeter calibrated using a 0.06% (+)-10-camphorsulfonic acid solution. Instrument optics and sample chamber were continuously flushed with 20 liters/min of dry N_2 gas. The purified and lyophilized 32 kDa enamelins were dissolved in 20 mM Tris (hydroxymethyl)aminomethane (Tris) buffer at pH 7.4 with an ionic strength (IS) 0.15 (0.15 M NaCl) 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 8 scans. The CD spectra of enamelins interactions with Ca^{2+} (20 mM Tris, pH 7.4, 0.15 M NaCl) were taken after 30-minute incubation at room temperature. CD spectra were expressed as the mean residue ellipticity, $[\theta]_{\text{MRW}}$, ($\text{deg cm}^2 \text{ dmol}^{-1}$), and $[\theta]_{\text{MRW}}$ 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 residual weight which is defined as the $M/N-1$ where M is the molecular mass ($M_{\text{enamelins}} = 32000 \text{ Da}$) and N is the number of amino acid residues, 'l' is the optical path length, and 'c' is the concentration of the protein (mg/ml). The secondary structure contents of the 32 kDa enamelins from the CD spectra were estimated by a FORTRAN program called CDSSTR

(<http://lamar.colostate.edu/~sreeram/CDPro/>) (Sreerama et al., 2000; Sreerama and Woody, 2000). The reference basis set used in this study is SP43, which contains the CD spectra of 43

soluble proteins with secondary structures obtained from high quality X-ray diffraction data. The accuracy of CD analysis was accessed based on the small values of root mean square deviation (δ). The tertiary class of the 32 kDa enamelins was also determined by running a program CLUSTER (Sreerama and Woody, 2000).

The calcium binding affinity (K_a) of the 32 kDa enamelins was determined from the fitting curve of the molar ellipticity at 222 nm. The $[\theta]_{mrw}$ at 222nm was plotted against Ca^{2+} concentration and analyzed by non-linear fitting curve procedures to yield K_a (Anderson et al., 1997).

2.5 ATR-FTIR spectroscopy

Fourier transform infrared (FTIR) spectra of porcine 32 kDa enamelins (0.17 mg/ml) in Tris buffer (pH 7.4, IS 0.15) and in the presence of 2.0 mM Ca^{2+} were recorded by using a JASCO FT 4100 spectrometer equipped with a fast recovery TGS detector. The attenuated total reflection (ATR) technique with a germanium crystal at 45° angle phase was utilized. A total of 128 scans at a resolution of 4 cm^{-1} were collected for each spectrum at room temperature. Difference spectra were obtained by the subtraction of the aqueous buffer from the corresponding spectrum of enamelins solution. The broad amide bands were analyzed using deconvolution and second derivative techniques. Fourier self-deconvolution (Yang et al., 1985) and derivative calculations (Susi and Byler, 1983; Lee et al., 1985) provide accurate assessments of the number and position of component peaks. Minima peaks in the second-derivative correspond to the positive absorptions in the original difference spectrum. Deconvolution of spectra was performed using the JASCO ANALYSIS function with a typical band width of 8 cm^{-1} and a resolution enhancement factor of 2.5. Second derivative spectra were calculated over an 11 data-point range (11 cm^{-1}).

3. Results

3.1 Characterization of the 32 kDa enamelins

The purity of extracted 32 kDa enamelins was above 95%, as confirmed by RP-HPLC (C4 analytical column) and by coomassie brilliant blue staining in SDS-PAGE (Fig. 1A). A blue band with a molecular weight around 32 kDa was observed in stains-all staining and this stains-all positive response is typical to the phosphorylated glycoprotein. The N-terminal sequence of the purified protein is LPHVPH-IPP, matching with the primary structure of the 32 kDa enamelins in the literature (Fig. 1B) (Hu and Yamakoshi, 2003).

3.2 Secondary Structure of the 32 kDa Enamelins Analyzed by CD and ATR-FTIR Spectroscopy

The far-UV CD spectrum of the 32 kDa enamelins in Tris buffer (pH 7.4, IS 0.15) shows two negative troughs at 207 nm with a mean residue ellipticity of $-3.0 \times 10^3 \text{ deg cm}^2 \text{ dmol}^{-1}$ and at 220 nm with $-2.3 \times 10^3 \text{ deg cm}^2 \text{ dmol}^{-1}$, and a positive maximum around 192 nm of $3.3 \times 10^3 \text{ deg cm}^2 \text{ dmol}^{-1}$ (Fig. 2A), which are characteristics of the secondary structure of proteins having a high content of α -helix (Kamal and Behere, 2002). Quantitative estimation for the secondary structural fractions of the 32 kDa enamelins by the program CDSSTR showed that it has 81.5% α -helix content, 10.1% β -sheet, 1.5% β -turns and 8.0% other structures (Table 1). The tertiary class of the 32 kDa enamelins determined by the program CLUSTER belongs to an Alfa+Beta class.

The FTIR absorption difference spectrum of the 32 kDa enamelins shows two main bands: the amide I and amide II maxima at 1655 and 1536 cm^{-1} respectively, and two minor bands at 1741 cm^{-1} and 1582 cm^{-1} (Fig. 2B). The amide bands contain a number of absorptions as revealed by the deconvolution and second-derivative spectra presented in Fig. 2C and 2D, respectively. It further showed a shoulder at 1671 cm^{-1} and a weak absorption at 1614 cm^{-1}

in the amide I region and a peak at 1513 cm^{-1} in the amide II band from the deconvolution and second derivative analysis. The major amide I component (1655 cm^{-1}) can be assigned to α -helical and/or random coil structures (Haris et al., 1986; Hadden et al., 1994). Other amide I components at 1614 cm^{-1} region are associated with β -sheet while the shoulder at 1671 cm^{-1} is assigned to an anti-parallel β -sheet (Haris et al., 1986; Hadden et al., 1994). The amide II maxima at 1536 cm^{-1} can not be assigned unambiguously to any particular secondary structure, but the absorption at 1513 cm^{-1} is related to the vibration of the tyrosine side chains (Hadden et al., 1994). The component at 1741 cm^{-1} is attributed to stretching vibration from the carboxyl (COO^-) groups while the band at 1582 cm^{-1} could be related to asymmetric stretching vibration of COO^- groups (Venjaminov and Kalnin, 1990).

3.3 Conformational changes of the 32 kDa enamel as a function of Ca^{2+}

The CD spectra show that sequential increase of CaCl_2 (0.05-10.0 mM) resulted in a progressive decrease in intensity of the minima at 208 nm and 220 nm and the maximum at 192 nm (Fig. 3A). At 0.05 mM Ca^{2+} , the $[\theta]_{\text{mrw}}$ of the two troughs at 207 nm and 220 nm are $-2.9 \times 10^3\text{ deg cm}^2\text{ dmol}^{-1}$ and $-2.2 \times 10^3\text{ deg cm}^2\text{ dmol}^{-1}$, respectively, which is slightly different from the data before Ca^{2+} was added. However, the $[\theta]_{\text{mrw}}$ at 192 nm substantially changed from $3.3 \times 10^3\text{ deg cm}^2\text{ dmol}^{-1}$ to $2.3 \times 10^3\text{ deg cm}^2\text{ dmol}^{-1}$. When CaCl_2 was increased to 2.0 mM, the troughs are slightly shifted to 208 nm with decreased intensity $-1.4 \times 10^3\text{ deg cm}^2\text{ dmol}^{-1}$ and to 221 nm of $-1.0 \times 10^3\text{ deg cm}^2\text{ dmol}^{-1}$. This blue shift is also observed for the positive band from 192 nm to 193 nm at 2.0 mM Ca^{2+} . At higher concentrations of Ca^{2+} (5.0 and 10 mM), there was no further changes in the CD spectra. A careful examination of the CD spectra reveals the presence of an isodichroic point at 200 nm, indicating structural transitions from α -helix to β -sheet, β -turns and other structures and a strong support to the conformational changes of the 32 kDa enamel as a function of Ca^{2+} concentration. CDSSTR analyses indicate that at an initial 0.05 mM Ca^{2+} , the α -helix content of enamel decreased from 81.5% to 68.0% with a concomitant increase of β -sheet from 10.1% to 13.8% and β -turns from 1.5% to 7.4%. With higher Ca^{2+} concentrations, the α -helix content of enamel decreased progressively while the β -sheet, the β -turns, and other structures increased simultaneously (Table 1). Such quantitative analysis clearly demonstrates that Ca^{2+} binding to the 32 kDa enamel decreased its α -helix content but increased its β -sheet, β -turns, and other structures, suggesting a preference of β -sheet conformation of the enamel in the presence of Ca^{2+} . The calcium association constant (K_a) of the 32 kDa enamel was calculated from the fitting curve of the $[\theta]_{\text{mrw}}$ at 222 nm (Fig. 3B). The determined K_a of the 32 kDa enamel is $1.55 (\pm 0.13) \times 10^3\text{ M}^{-1}$, which is in good agreement with the value of $5.2 \times 10^3\text{ M}^{-1}$ (Yamakoshi et al., 2001), indicating a relatively weak affinity of enamel to calcium ions.

In the presence of 2.0 mM Ca^{2+} , FTIR difference spectrum of the 32 kDa enamel solution apparently shows the broadening of amide I band, the weakening of the amide II region and the shift of absorption of the COO^- groups (Fig. 3C). The deconvolution and second-derivative spectra of enamel with Ca^{2+} (Fig. 3D and 3E) show that in addition to the shoulders at 1672 cm^{-1} and 1614 cm^{-1} , a new absorption appears at 1638 cm^{-1} , which is associated with β -sheet (Haris et al., 1986; Hadden et al., 1994). The presence of the new absorption and the broadening of the amide I band are in good agreement with the increase of β -sheet conformation upon the addition of Ca^{2+} in the CD study. The intensity of the amide II band weakened and it became broad at $1539\text{--}1543\text{ cm}^{-1}$ at 2.0 mM Ca^{2+} . It is also noticeable that the intensity of the asymmetrical vibration of COO^- group at 1582 cm^{-1} is significantly reduced while the symmetrical stretching mode shifts to $1749\text{--}1765\text{ cm}^{-1}$ and becomes broadened, clearly as a result of Ca^{2+} binding to COO^- groups.

4. Discussion

Based on identified mutations in *ENAM* gene in cases of *amelogenesis imperfecta* (AI) (Pavlič et al., 2007; Hu et al., 2003), the randomly-induced point mutations in animal models resembling these clinical cases, and the recent reports on *Enam* -null mice (Hart et al., 2003; Masuya et al., 2005, Hu et al 2008), it is obvious that the presence of enamelin protein is absolutely critical for normal enamel formation. In many of these cases the mutation occurs in regions associated with the sequence of the 32 kDa fragment highlighting the importance of this segment in controlling enamel mineralization (Masuya et al., 2005). A few in vitro mineralization studies have provided evidence that the 32 kDa enamelin interacts with apatite crystals, suggesting direct involvement of enamelin in the processes of crystal nucleation and growth (Tanabe et al., 1990; Bouropoulos and Oldak, 2004, Hu et al., 2008). Here, in order to further clarify the function of enamelin protein during enamel biomineralization, we investigated the structural properties of the 32 kDa enamelin and analyzed its conformational changes as a function of Ca^{2+} .

4.1 Analysis of secondary structure of the 32 kDa enamelin

CD data showed that despite the presence of 18.8% proline in the 32 kDa enamelin, this glycoprotein is rich in α -helix conformation. The high content α -helix structure is possibly arising from the effect of the post-translational modifications of enamelin (Yamakoshi, 1995). When compared with traditional α -helix conformation having two minima at 208 nm and 222 nm, the trough of the 32 kDa enamelin at 220 nm is a little flat and slightly red shifted, which is likely due to the contribution from 10.1% β -sheet conformation. It is apparent that the CD spectrum of the 32 kDa enamelin is different from that of the principal enamel protein amelogenin, which has a high percentage of polyproline type II helix and unordered structures, as manifested by an earlier study (Renugoplakrishnan et al., 1986; Lakshminarayanan et al., 2007). This elliptically polarized difference indicates that the 32 kDa enamelin and rP172 amelogenin have their distinctive secondary structures and consequently may play different but complimentary roles during enamel formation. In a good agreement with the CD results, FTIR spectra also indicated a characteristic absorption of a protein containing high α -helix conformation (Fig. 2B-D).

4.2 Calcium effect on the conformational changes of the 32 kDa enamelin

Quantitative analysis of the secondary structure fractions of the 32 kDa enamelin revealed that it has a conformational preference to β -sheet upon addition of Ca^{2+} at concentration range comparable to those measured in the enamel fluid (Aoba and Moreno, 1987). It apparently results from Ca^{2+} binding to carboxyl and phosphate side groups of the 32 kDa enamelin, leading to a stabilization of β -sheet conformation and a changing of the CD of the backbone amides in the ultraviolet region. These changes of CD data demonstrate that the 32 kDa enamelin has a tendency to favor the binding of Ca^{2+} through ionic interactions. Studies have shown that other calcium-binding proteins, such as calmodulin, undergo conformational changes to a well-ordered structure upon the coordination of Ca^{2+} (Meador et al., 1992; Finn et al., 1995). Calcium binding induced conformational changes has been reported in both low and high affinity Ca^{2+} -binding motifs in proteins (Engel et al., 1987; Nelson and Chazin, 1998; Ababou et al., 2001; Yang and Klee 2000). Polycarboxylic acid polymers such as poly-aspartic acid also assume β -sheet conformation in the presence of calcium (Keith, 1971; Addadi et al., 1991). In an in vitro model system using calcium fumarate crystals grown in the presence of poly-ASP, Addadi et al have shown that the β -sheet conformation is important for allowing effective interactions to occur between macromolecules and growing crystals (Addadi and Weiner, 1989; Addadi et al., 1991). FTIR measurements in the presence of Ca^{2+} showed the broadening of amide I band, the shifting of the COO^- group absorption in the enamelin difference spectrum, and the appearance of a new band at 1638 cm^{-1} in the deconvoluted and

second derivative spectra. Such absorption changes likely arise from the ionic interactions between Ca^{2+} and COO^- groups, which are in line with the CD results of the decrease of α -helix and the increase of β -sheet conformation of the 32 kDa enamelin as a function of Ca^{2+} .

It has been reported that the phosphorylated glycoprotein 32 kDa enamelin adsorb strongly onto apatite crystals (Tanabe et al., 1990). The affinity of the 32 kDa enamelin to Ca^{2+} is weaker than the 13, 15, 27, 29 kDa proteins cleaved from the C-terminus of sheathlin but stronger than the amelogenin proteins (Yamakoshi et al., 2001). Given the fact that these proteins have low abundance in the enamel matrix, they may function as nucleation sites and substantially promote the apatite crystal growth with the cooperation with the dominant amelogenins (Bouropoulos and Oldak, 2004). Alternatively, these proteins would be expected to buffer the free calcium ion concentration in the enamel fluid (Aoba and Moreno, 1987). Considering the 32 kDa enamelin (g.8344delG) mutation causing hypoplastic enamel of AI, the calcium effect to enamelin conformation demonstrates an important functional role during the normal enamel formation.

In summary, by applying spectroscopical techniques we report conformational changes of the 32 kDa enamelin as a function of Ca^{2+} . The precise nature of the conformational transition occurring at various concentrations of Ca^{2+} and the relationship between the conformational changes of enamelin with its self-association and its function needs to be studied. We speculate that enamelin may cooperate closely with amelogenin in controlling mineral growth during enamel formation and work is in progress to investigate direct interactions between amelogenin and enamelin. 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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References

- Ababou A, Shenvi RA, Desjarlais JR. Long-range effects on calcium binding and conformational change in the N-domain of calmodulin. *Biochemistry* 2001;40:12719–12726. [PubMed: 11601997]
- Addadi, L.; Moradian-Oldak, J.; Weiner, S. ACS Symposium Series; 1991. p. 13-27.
- Addadi, L.; Weiner, S. *Biomaterialization Chemical and Biochemical Perspectives*. Mann, S.; Webb, J.; Williams, RJP., editors. VCH Verlagsgesellschaft; Weinheim: 1989. p. P133
- Anderson LA, Palmer T, Price NC, Bornemann S, Boxer DH, Pau RN. Characterization of the molybdenum-responsive ModE regulatory protein and its binding to the promoter region of the *modABCD* (molybdenum transport) operon of *Escherichia coli*. *Eur. J. Biochem* 1997;246:119–126. [PubMed: 9210473]
- Aoba T, Moreno EC. The enamel fluid in the early secretory stage of porcine amelogenesis: Chemical composition and saturation with respect to enamel mineral. *Calcif. Tissue Int* 1987;41:86–94. [PubMed: 3115550]
- Beniash E, Simmer JP, Margolis HC. The effect of recombinant mouse amelogenins on the formation and organization of hydroxyapatite crystals in vitro. *J. Struct. Biol* 2005;149:182–190. [PubMed: 15681234]
- Bouropoulos N, Moradian-Oldak J. Induction of apatite by the cooperative effect of amelogenin and the 32-kDa enamelin. *J. Dent. Res* 2004;83:278–282. [PubMed: 15044499]
- Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem* 1976;72:248–254. [PubMed: 942051]

- Caday CG, Lambooy PK, Steiner RF. The interaction of Ca^{2+} -binding proteins with the carbocyanine dye stains-All. *Biopolymers* 1986;25:1579–1595. [PubMed: 2427135]
- Campbell KP, MacLennan DH, Jorgensen AO. Staining of the Ca^{2+} -binding proteins, calsequestrin, calmodulin, troponin C, and S-100, with the cationic carbocyanine dye “Stainsall”. *J. Biol. Chem* 1983;258:11267–11273. [PubMed: 6193121]
- Dohi N, Murakami C, Tanabe T, Yamakoshi Y, Fukae M, Yamamoto Y. Immunocytochemical and immunochemical study of enamelin, using antibodies against porcine 89-kDa enamelin and its N-terminal synthetic peptide, in porcine tooth germs. *Cell Tissue Res* 1998;293:313–325. [PubMed: 9662654]
- Du C, Falini G, Fermani S, Abbott C, Moradian-Oldak J. Supramolecular assembly of amelogenin nanospheres into birefringent microribbons. *Science* 2005;307:1450–1454. [PubMed: 15746422] Erratum. *Science* 309, 2166
- Engel J, Taylor W, Paulsson M, Sage H, Hogan B. Calcium binding domains and calcium-induced conformational transition of SPARC/BM-40/Osteonectin, an extracellular glycoprotein expressed in mineralized and nonmineralized tissues. *Biochemistry* 1987;26:6958–6965. [PubMed: 3427055]
- Finn BE, Evenas J, Drakenberg T, Waltho JP, Thulin E, Forsens X. Calcium-induced structural changes and domain autonomy in calmodulin. *Nat. Struct. Biol* 1995;2:777–783. [PubMed: 7552749]
- Fukae M, Tanabe T, Murakami C, Dohi N, Uchida T, Shimizu M. Primary structure of the porcine 89-kDa enamelin. *Adv. Dent. Res* 1996;10:111–118. [PubMed: 9206327]
- Gibson CW, Yuan ZA, Hall B, Longenecker G, Chen E, Thyagarajan T, Sreenath T, Wright JT, Decker S, Piddington R, Harrison G, Kulkarni AB. Amelogenin-deficient mice display an amelogenesis imperfecta phenotype. *J. Biol. Chem* 2001;276:31871–31875. [PubMed: 11406633]
- Habelitz S, Kullar A, Marshall SJ, DenBesten PK, Balooch M, Marshall GW, Li W. Amelogenin-guided crystal growth on fluoroapatite glass-ceramics. *J. Dent. Res* 2004;83:698–702. [PubMed: 15329375]
- Hadden JM, Bloemendal M, Haris PI, Srai SKS, Chapman D. Fourier transform infrared spectroscopy and differential scanning calorimetry of transferrins: human serum transferrin, rabbit serum transferrin and human lactoferrin. *Biochim. Biophys. Acta* 1994;1205:59–67. [PubMed: 8142485]
- Haris PI, Lee DC, Chapman D. Determination of protein secondary structure using factor analysis of infrared spectra. *Biochim. Biophys. Acta* 1986;874:255–265. [PubMed: 3790572]
- Hart PS, Michalec MD, Seow WK, Hart TC, Wright JT. Identification of the enamelin (g.8344delG) mutation in a new kindred and presentation of a standardized ENAM nomenclature. *Arch. Oral Biol* 2003;48:589–596. [PubMed: 12828988]
- Hu CC, Hu Y, Smith CE, Mckee MD, Wright JT, Yamakoshi Y, Papagerakis P, Hunter GK, Feng JQ, Yamakoshi F, Simmer JP. Enamel defects and ameloblast-specific expression in *enamelin* knockout/Lacz knockin mice. *J. Biol. Chem* 2008;283in press
- Hu CC, Yamakoshi Y. Enemelin and autosoml-dominant amelogenesis imperfecta. *Crit. Rev. Oral. Biol. Med* 2003;14:387–398. [PubMed: 14656895]
- Hu CC, Zhang CH, Ying Y, Karrman-Mardh C, Forsman-Semband K, Simmer JP. Cloning and characterization of the mouse and human enamelin genes. *J. Dent. Res* 2001;80:898–902. [PubMed: 11379892]
- Hu CC, Hart TC, Dupont BR, Chen JJ, Sun X, Qian Q, Zhang CH, Jiang H, Mattern VL, Wright JT, Simmer JP. Cloning human enamelin cDNA, chromosomal localization, and analysis of expression during tooth development. *J. Dent. Res* 2000;79:912–919. [PubMed: 10831092]
- Hu CC, Simmer JP, Bartlett JD, Qian Q, Zhang C, Ryu OH, Xue J, Fukae M, Uchida T, MacDougall M. Murine enamelin: cDNA and derived protein sequences. *Connect. Tiss. Res* 1998;39:351–365.
- Hu CC, Fukae M, Uchida T, Qian Q, Zhang CH, Ryu OH, Tanabe T, Yamakoshi Y, Murakami C, Dohi N, Shimizu M, Simmer JP. Cloning and characterization of porcine enamelin mRNAs. *J. Dent. Res* 1997;76:1720–1729. [PubMed: 9372788]
- Iijima M, Moradian-Oldak J. Control of octacalcium phosphate and apatite crystal growth by amelogenin matrices. *J. Mat. Chem* 2004;14:2189–2199.
- Kamal JK, Behere DV. Thermal and conformational stability of seed coat soybean peroxidase. *Biochemistry* 2002;41:9034–9042. [PubMed: 12119017]
- Keith HD. *Biopolymers* 1971;10:1099–1105.

- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685. [PubMed: 5432063]
- Lakshminarayanan R, Fan D, Du C, Moradian-Oldak J. *Biophys. J* 2007;93:3664–3674. [PubMed: 17704165]
- Lee DC, Hayward JA, Restall CJ, Chapman D. Second-derivative infrared spectroscopic studies of the secondary structures of bacteriorhodopsin and Ca^{2+} -ATPase. *Biochemistry* 1985;24:4364–4373. [PubMed: 2932156]
- Margolis HC, Beniash E, Fowler CE. Role of macromolecular assembly of enamel matrix proteins in enamel formation. *J. Dent. Res* 2006;85:775–793. [PubMed: 16931858]
- Masuya H, Shimizu K, Sezutsu H, Sakuraba Y, Nagano J, Shimizu A, Fujimoto N, Kawai A, Miura I, Kaneda H, Kobayashi K, Ishijima J, Maeda T, Gondo Y, Noda T, Wakana S, Shiroishi T. Enamelin (*Enam*) is essential for amelogenesis: ENU-induced mouse mutants as models for different clinical subtypes of human amelogenesis imperfecta (AI). *Hum. Mol. Genet* 2005;14:575–583. [PubMed: 15649948]
- Meador WE, Means AR, Quijcho FA. Target enzyme recognition by calmodulin: 2.4 Å structure of a calmodulin-peptide complex. *Science* 1992;257:1251–1255. [PubMed: 1519061]
- Moradian-Oldak, J.; Paine, ML. Mammalian Dental Enamel. In: Sigel, HSA.; Sigel, RKO., editors. *Biom mineralization. From Nature to Application*. John Wiley & Sons, Ltd; Chichester: 2008. in Press
- Moradian-Oldak J, Du C, Falini G. On the formation of amelogenin microribbons. *Eur. J. Oral Sci* 2006;114(Suppl 1):289–96. [PubMed: 16674701]
- Nelson MR, Chazin WJ. An interaction-based analysis of calcium-induced conformational changes in Ca^{2+} sensor proteins. *Protein Science* 1998;7:270–282. [PubMed: 9521102]
- Paine ML, White SN, Luo W, Fong H, Sarikaya M, Snead ML. Regulated gene expression dictates enamel structure and tooth function. *Matrix Biol* 2001;20:273–292. [PubMed: 11566262]
- Pavlič A, Petelin M, Battelino T. Phenotype and enamel ultrastructure characteristics in patients with ENAM gene mutations g.13185-13186insAG and 8344delG. *Arch. Oral Biol* 2007;52:209–217. [PubMed: 17125728]
- Renugoplakrishnan V, Strawich ES, Horowitz PM, Glimcher MJ. Studies of the secondary structures of amelogenin from bovine tooth enamel. *Biochemistry* 1986;25:4879–4887. [PubMed: 3768319]
- Robinson, C.; Kirkham, J.; Bonass, WA.; Shore, R.; Brookes, SJ. Dental Enamel: Formation to Destruction. CRC Press, Inc.; Boca Raton, Florida: 1995. p. 105-134. Chapter 5
- Sreerama N, Venyaminov SY, Woody RW. Estimation of protein secondary structure from circular dichroism spectra: inclusion of denatured proteins with native proteins in the analysis. *Anal. Biochem* 2000;287:243–251. [PubMed: 11112270]
- Sreerama N, Woody RW. Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. *Anal. Biochem* 2000;287:252–260. [PubMed: 11112271]
- Susi H, Byler DM. Protein structure by Fourier transform infrared spectroscopy: second derivative spectra. *Biochem. Biophys. Res. Commun* 1983;115:391–397. [PubMed: 6615537]
- Tanabe T, Aoba T, Moreno EC, Fukae M, Shimizu M. Properties of phosphorylated 32 kd nonamelogenin proteins isolated from porcine secretory enamel. *Calcif. Tissue Int* 1990;46:205–215. [PubMed: 2106381]
- Uchida T, Tanabe T, Fukae M, Shimizu M. Immunocytochemical and immunochemical detection of a 32 kDa non-amelogenin and related proteins in porcine tooth germs. *Arch. Histol. Cytol* 1991;54:527–538. [PubMed: 1793666]
- Venyaminov SY, Kalnin NN. Quantitative IR spectrophotometry of peptide compounds in water (H_2O) solutions. I. Spectral parameters of amino acid residue absorption bands. *Biopolymers* 1990;30:1243–1257. [PubMed: 2085660]
- Yamakoshi Y. Carbohydrate moieties of porcine 32 kDa enamel. *Calcif. Tissue Int* 1995;56:323–330. [PubMed: 7767845]
- Yamakoshi Y, Tanabe T, Fukae M, Shimizu M. Porcine amelogenins. *Calcif. Tissue Int* 1994;54:69–75. [PubMed: 8118757]

- Yamakoshi Y, Tanabe T, Oida S, Hu CC, Simmer JP, Fukae M. Calcium binding of enamel proteins and their derivatives with emphasis on the calcium-binding domain of porcine sheathlin. *Arch. Oral Biol* 2001;46:1005–1014. [PubMed: 11543707]
- Yang S, Klee CB. Low affinity Ca^{2+} -binding sites of calcineurin B mediate conformational changes in calcineurin A. *Biochemistry* 2000;39:16147–16154. [PubMed: 11123943]
- Yang WJ, Griffiths PR, Byler DM, Susi H. Protein conformation by infrared spectroscopy: resolution enhancement by Fourier self-deconvolution. *Appl. Spectrosc* 1985;39:282–287.

(B) ¹⁷⁴LWHVPHRIPP GYGRPPTSNE EGGNPYFGFF GYHGFGRPP YYSEEMFEQD
FEKPKEKDPP KTETPATEPS VNTTVPETNS TQPNAPNPRG NDTSPGTSG QGPNPR²⁷⁹

Fig. 1. Characterization of the purified 32 kDa enamelin. (A) RP-HPLC elution profile after purification with first a C4 and then a C18 column. SDS-PAGE gel of the purified protein stained with stains-all (SA) and coomassie brilliant blue (CBB). Note that lane SA is the protein isolated after C4 column and lane CBB is after purification by C18 column. (B) Amino acid sequence of the 32 kDa enamelin.

Fig. 2. Secondary structure of the 32 kDa enamelin (0.17 mg/ml in 20mM Tris buffer, pH 7.4, 0.15 M NaCl). (A) CD spectrum. (B) ATR-FTIR absorption difference spectrum. (C) Deconvoluted spectrum. (D) Second-derivative spectrum.

Fig. 3. Conformational changes of the 32 kDa enamelin as the function of Ca^{2+} . (A) CD spectra of the 32kDa enamelin in the presence of different concentration of Ca^{2+} (mM). (B) The fitting curve of ellipticity at 222 nm and determined calcium binding affinity (K_a) of enamelin. (C) ATR-FTIR absorption difference spectrum in the presence of 2.0 mM Ca^{2+} . (D) Deconvoluted spectrum. (E) Second-derivative spectrum.

Table 1

Quantitative analysis of secondary structure of the 32 kDa enamelin (20 mM Tris, pH 7.4, 0.15 M NaCl) as a function of Ca^{2+} .
Program: CDSSTR; Ref. Protein Set: SP43

Ca^{2+} Con.(mM)	α -Helix	β -Sheet	Turns	Unknown	δ^a
0	81.5%	10.1%	1.5%	8.0%	0.053
0.05	68.0%	13.8%	7.4%	10.2%	0.071
0.2	64.5%	15.2%	6.8%	13.4%	0.062
0.5	62.6%	16.7%	5.9%	14.8%	0.046
1.0	58.7%	17.5%	8.1%	15.3%	0.071
2.0	53.0%	18.0%	13.3%	16.3%	0.050

^a δ , root mean square deviation.