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Ameloblastin peptide encoded by exon 5 interacts with amelogenin N-terminus

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ABSTRACT

Interactions between enamel matrix proteins are important for enamel biomineralization. In recent *in situ* studies, we showed that the N-terminal proteolytic product of ameloblastin co-localized with amelogenin around the prism boundaries. However, the molecular mechanisms of such interactions are still unclear. Here, in order to determine the interacting domains between amelogenin and ameloblastin, we designed four ameloblastin peptides derived from different regions of the full-length protein (AB1, AB2 and AB3 at N-terminus, and AB6 at C-terminus) and studied their interactions with recombinant amelogenin (rP172), and the tyrosine-rich amelogenin polypeptide (TRAP). A series of amelogenin Trp variants (rP172(W25), rP172(W45) and rP172(W161)) were also used for intrinsic fluorescence spectroscopy. Fluorescence spectra of rP172 titrated with AB3, a peptide encoded by exon 5 of ameloblastin, showed a shift in λ_{max} in a dose-dependent manner, indicating molecular interactions in the region encoded by exon 5 of ameloblastin. Circular dichroism (CD) spectra of amelogenin titrated with AB3 showed that amelogenin was responsible for forming α-helix in the presence of ameloblastin. Fluorescence spectra of amelogenin Trp variants as well as the spectra of TRAP titrated with AB3 showed that the N-terminus of amelogenin is involved in the interaction between ameloblastin and amelogenin. We suggest that macromolecular co-assembly between amelogenin and ameloblastin may play important roles in enamel biomineralization.

1. Introduction

The highly ordered structure of tooth enamel is regulated by the cells and extracellular matrix proteins that work together to control crystal initiation and organized growth [1]. These enamel matrix proteins are secreted by ameloblasts during the secretory stage of amelogenesis to create a mineralization front where the enamel crystals grow in length. The matrix proteins are degraded during the maturation stage by proteinases, and eventually removed as the enamel crystals grow in width and thickness [2,3].

The major structural proteins in enamel are amelogenin (Amel), ameloblastin (Ambn), amelotin (Amtn) and enamelin (Enam) [1]. Amelogenin is the most abundant, accounting for approximately 90% of the enamel matrix protein. Mutations in the *amelogenin* gene result in X-linked *amelogenesis imperfecta* [4,5]. In 16-week-old mice, amelogenin deficiency resulted in an enamel thickness of $<10\%$ of normal enamel, with disorganized prism structure [6]. Ameloblastin is the second most abundant protein, accounting for roughly 5% of enamel matrix protein [7]. Genomic deletion of *AMBN* exon 6 in humans results in hypoplastic *amelogenesis imperfecta* [8]. In the *Ambn* mutant mouse that expressed a truncated Ambn variant without the segment encoded by exons 5 and 6, ameloblasts detached from the tooth surface at the early secretory stage, and the mutation resulted in defective enamel formation [9,10]. *In vitro* experiment further showed that the segment encoded by exon 5 may be involved in ameloblastin self-assembly [11].

Evidence for the notion that amelogenin and ameloblastin may have cooperative function was provided by recent double knock out animal model studies [10]. In 7-day-old *Amel* $X^{-/-}$/*Ambn*$^{-/-}$ mice, the ameloblast layer was irregular and detached from the enamel surface as in *Ambn*$^{-/-}$ mice, and the enamel width was significantly thinner than that in *Amel* $X^{-/-}$ or *Ambn*$^{-/-}$ mice [12]. In a different report, dual-immunogold labeling showed the co-distribution of ameloblastin and amelogenin, suggesting a functional association between these two proteins [13]. It has been shown that the amelogenin trityrosyl-motif peptide localized at the N-terminal region of amelogenin binds to ameloblastin [14]. Immunochemical assays revealed that ameloblastin co-localized with amelogenin during the early stages of tooth development [15].

Abbreviations: Amel, Amelogenin; Ambn, Ameloblastin; TRAP, Tyrosine Rich Amelogenin Polypeptide; CD, Circular Dichroism; HPLC, High Performance Liquid Chromatography

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Recently, using an advanced quantitative co-localization assay, we reported that amelogenin co-localized with ameloblastin at the mineralization front during the secretory stage of enamel formation [16]. We further demonstrated *in vitro* that addition of recombinant full-length amelogenin to recombinant full-length ameloblastin induced conformational change in the latter, hinting at intermolecular interactions between the two proteins [16]. Our recent *in vivo* study further shows that the N-terminal proteolytic product of ameloblastin co-localizes with the N-terminus of amelogenin around the prism boundary [17]. However, it is not known how these proteins interact and whether any structural changes result from the interaction. In order to provide additional support for direct amelogenin-ameloblastin interactions and to determine the interacting domains between them, we designed four ameloblastin peptides derived from different regions of the full-length protein. We used intrinsic fluorescence spectroscopy and circular dichroism to study their interaction with amelogenin and the N-terminal tyrosine-rich amelogenin polypeptide (TRAP) [18].

2. Materials and methods

2.1. Wild type and variant Amelogenin expression and purification

Wild type (rP172) and variant recombinant porcine amelogenin proteins were expressed in *E. coli* strain BL21(DE3)pLysS (Stratagene, CA), and precipitated by 20% ammonium sulfate following the method described previously [19,20]. The ammonium sulfate precipitate was dissolved in water containing 0.1% TFA and purified using a high performance liquid chromatography (HPLC) system (Varian, CA) equipped with a Phenomenex C4 column (10×250 mm, 5 μm). The purified proteins were lyophilized, kept at −20 °C, and dissolved in water before use. Our group designed three double-variant strains of amelogenin; rP172(W45Y, W161Y) will be referred to as rP172(W25), rP172(W25Y, W161Y) will be referred to as rP172(W45), and rP172(W25Y, W45Y) will be referred to as rP172(W161) [21]. The positions of tryptophan residues of rP172 and its variants are illustrated in Table 1.

2.2. Peptide synthesis

Four peptides (AB1, AB2, AB3, and AB6) were designed based on the amino acid sequence of mouse ameloblastin (Ambn), and synthesized by Chempeptide Limited (Shanghai, China). AB1 includes 40 amino acid residues encoded by exon 3 and 4, AB2 is next to AB1 and includes 37 amino acid residues encoded by exon 5, AB3 is a variant of AB2 with two tryptophan residues substituted by two tyrosine residues, and AB6 includes 44 amino acid residues encoded by exon 13 and located at the C-terminus of Ambn. AB3 has no tryptophan residue, which is a fluorophore, and it is used instead of AB2 in the experiments where amelogenin-ameloblastin interactions are studied. The purity of peptides was determined by HPLC equipped with Kromasil-C18 column (4.6×250 mm, 5 μm), and the concentration was determined by Pierce BCA protein assay kit (Thermo Scientific, IL).

TRAP consisting of 45 amino acid residues at the N-terminus of full-length mouse amelogenin was synthesized at USC Microchemical Core Facility as previously described [22]. The amino acid sequences of AB1, AB2, AB3, AB6, and TRAP are shown in Table 2. The two substituted tyrosine residues are labeled in red.

2.3. Intrinsic fluorescence spectroscopy

Lyophilized amelogenin and peptides were first dissolved in water to make stock solutions with higher concentration of protein or peptide. All the samples were prepared by diluting the protein or the peptide into buffer (5 mM Tris-HCl) at pH of 7.3. Table 2 summarizes the molecular masses of the synthetic peptides.

Table 1
The tryptophan residues of wild type amelogenin and its variants.

Name	Location of Tryptophan residues		
rP172	W^{25}	W^{45}	W^{161}
rP172(W25)	W^{25}		
rP172(W45)		W^{45}	
rp172(W161)			W^{161}

To investigate whether amelogenin interacts with ameloblastin, samples containing 10 μM rP172 and 0, 5, 10, or 15 μM AB1, 10 μM rP172 and 0, 5, 10, or 15 μM AB3, and 10 μM rP172 and 0, 5, 10, or 15 μM AB6 were prepared separately. For collecting fluorescence spectra of rP172 titrated with AB3, 10 μM rP172(W25) was mixed with 0, 5, 10, or 15 μM AB3, 10 μM rP172(W45) was mixed with 0, 5, 10, or 15 μM AB3, and 10 rP172(W161) was mixed with 0, 5, 10, or 15 μM AB3. For collecting fluorescence spectra of TRAP titrated with AB3, 10 μM TRAP was mixed with 0, 1, 5, or 10 μM AB3. After preparation, all samples were kept at room temperature for 1 h before measurement.

The fluorescence emission spectra were collected using a QuantaMaster 4 spectrofluorometer (Photon Technology International, NJ) equipped with a 1-cm path-length cuvette. The excitation wavelength was 295 nm, the window sizes of the excitation and the emission shutters were set to 5 nm, and the final spectra were the average of three scans. All fluorescence experiments were carried out at room temperature.

2.4. Circular dichroism (CD) spectroscopy

We mixed 2.5 μM of rP172 with 0, 1.25, 2.5 or 3.75 μM AB3 separately, and kept the solutions at room temperature for 1 h before measurement. The final solutions contained 5 mM Tris-HCl and their pH was 7.3. The CD spectra were collected using a J-815 spectrometer (Jasco, Japan) with a 1-mm path-length cuvette. The low concentration of Tris–HCl and short path-length reduced the effect of Tris–HCl at wavelength lower than 200 nm. The wavelength range was 190–250 nm. The final spectra were the average of three scans. The CD spectra of buffer with different concentrations of AB3 were collected first. These spectra were used as reference data, and were subtracted from the spectra of rP172 titrated with corresponding concentration of AB3. Finally, the CD spectra of rP172 in the presence of AB3 were collected and recorded. The experiments were carried out at room temperature.

3. Results

3.1. Identifying ameloblastin domains that interact with full-length amelogenin

In order to identify the Amel-interacting domains on Ambn, recombinant porcine Amel rP172 (which is an 89% analogue to mouse Amel) was titrated with peptides AB1, AB3 and AB6 at pH 7.3 5 mM Tris–HCl buffer (Fig. 1(a)). CD analysis confirmed that the secondary structures of AB3 are similar to those of the wild type sequence (AB2), suggesting that substitution of the two tryptophan residues did not affect folding (Fig. S1). Thus, AB3 was used instead of the wild type sequence of Abmn (AB2) which preserves two tryptophan residues (Table 2). When rP172 was titrated with the AB1 comprising the N-terminal 40 amino acid residues of