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Control of apatite crystal growth in a fluoride containing amelogenin-rich matrix

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Abstract

To study how crystal growth in dental enamel is controlled by the components of the extracellular matrix, we investigated the functional roles of amelogenins and fluoride ions in apatite formation occurring through an octacalcium phosphate (OCP)-precursor pathway. Using a cation selective membrane system as a model of tooth enamel formation, we evaluated the resulting mineral habit grown in native porcine amelogenins and fluoride ions. In the absence of amelogenin and in the presence of 1 or 2 ppm F, we obtained OCP + apatite and apatite, respectively. Without amelogenins, the crystals were hexagonal prisms and cones with diameters of ~100–200 nm. In the presence of 10% amelogenins and in the absence of fluoride, rod-like OCP with a diameter of 35 nm were obtained. Remarkably, a combination of amelogenin and fluoride created the formation of rod-like apatite crystals with dimensions similar to the former crystals. These observations indicate a cooperative role of amelogenin and fluoride in the regulation of habit, size orientation and phase of the calcium-phosphate crystals, resulting in the formation of fine rod-like apatite whose habit and orientation were similar to that of authentic tooth enamel crystals. The significant modulating effect of the amelogenin matrix combined with fluoride ions suggests the potential for this artificial system to contribute to the engineering of novel enamel-like biomaterials in vitro.

Keywords: Tooth enamel; Apatite; Amelogenins; Fluoride; Biomimetic material

1. Introduction

Dental enamel is a bioceramic composite made by a series of cell-mediated processes and is the hardest tissue in the vertebrate body. The mineralization of dental enamel starts with the deposition of very thin ribbon-like crystals in an amelogenin-rich extracellular matrix and terminates with a hard tissue, which is composed of highly oriented and densely packed prism-like crystals [1–4]. Mature enamel crystals have been characterized as carbonate-apatite [5–8], while the initial crystallite is not yet identified directly. Accumulation of numerous experimental evidence [9–18] has supported the hypothesis that enamel apatite formation involves octacalcium phosphate (OCP) as a precursor phase [10,11,14–16,18,19]. According to this proposed mechanism, enamel crystals initiate as thin ribbons of OCP-like crystallites elongated in their *c*-axis direction and have a thickness of one unit cell of OCP [14–19]. The overgrowth of apatite takes place on the (1 0 0) faces of the template OCP crystals, resulting in an increase in crystal thickness, and eventually, oriented and tightly assembled prisms are formed.

Amelogenins are the principal protein component of the enamel extracellular matrix, occupying more than 90% of the matrix during the developing stage of enamel formation [20,21]. Recent in vitro data has strongly supported the hypothesis that amelogenins play key roles as a scaffold controlling the growth of enamel crystals [22–24]. It has been shown that 1–2% porcine amelogenins incorporated in a gelatin gel resulted in the elongation of OCP crystals [25]. Recently we have demonstrated that amelogenin nanospheres change the morphology of OCP crystals from ribbon-like to prismatic and rod-like by interacting with specific side faces of OCP crystals [26–29]. These effects were

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ascribed to the hydrophobic nature of the amelogenin nanospheres.

The fluid of the enamel matrix contains several inorganic ions as minor components [30]. Among these ions, fluoride ions have substantial effect on apatite formation even at a very small concentration. It has been established that ppm levels of F accelerates transition of OCP to hydroxyapatite [31], improves the crystallinity of apatite [32] and induces F-containing hydroxyapatite $Ca_5(PO_4)_3OH_{1-x}F_x$ formation, which has a K_{sp} (6.5×10^{-65} for $x = 0.56$) [33] lower than hydroxyapatite ($K_{sp}(OHAp) = 7.36 \times 10^{-60}$ [33], pKs = 57.5 [34]). In our cation selective membrane system, $0.1 \sim 1$ ppm F induced epitaxial growth of F-containing apatite on the (1 0 0) face of OCP, resulting in apatite/OCP/apatite lamellar mixed crystals, which resembled an enamel apatite crystal both in appearance and structure [15,16,35]. Furthermore, F incorporated in the lattice enhanced adsorption of porcine amelogenin on fluoridated apatite $Ca_5(PO_4)_3OH_{1-x}F_x$ ($x > 0.1$) [36] and OCP-fluoridated hydrolyzate crystals [37,38].

We therefore hypothesize that during enamel apatite formation, amelogenins, OCP, and F are key parameters in controlling the growth of enamel crystallites. While our previous in vitro studies [25–29] have focused on the growth of OCP in amelogenin matrices, the present study aimed to grow apatite crystals. We seek to explore possible cooperative effect among the three parameters, namely amelogenin, fluoride, and OCP in controlling the orientation, habit and mineral phase of enamel crystals. For this purpose, using the model system for tooth enamel formation, crystal growth was carried out in native porcine amelogenins and in the presence of ppm levels of fluoride. The cation selective membrane device used in this study is an ideal system to mimic the enamel extracellular matrix when calcium ions are entered in a uni-directional flow in to the crystallization space.

2. Materials and methods

2.1. Selective extraction of amelogenins from porcine molars

Porcine amelogenins (P-Amel) were extracted following the dissociative extraction procedure of Termine et al. [20]. Enamel scrapings were collected from unerupted fourth and fifth mandibular molars of fresh six-month-old pig jaws. The pig mandibles were obtained fresh from a local slaughter house (Farmer John Clougherty Co., Los Angeles, CA) through Sierra For Medical Science, (Santa Fe Springs, CA). The United States Department of Agriculture has inspected and approved the process of sample harvesting. Amelogenins were extracted in Guanidine HCl (4M) - Tris (50 mM) at pH 7.4, desalted, and concentrated by Amicon ultrafiltration using a 10 kDa cutoff Amicon YM-10 membrane against 0.5% formic acid. The protein solution was lyophilized and stored at −20°C. The composition of this amelogenin-rich extract has been previously defined by SDS-gel and estimated to contain about 7% 25 kDa (P173, the full-length porcine amelogenin), 49% 20 kDa (P148), 23 kDa (10%), and 32% 18–10 kDa. For more details on the composition and structural characterization of the amelogenins used, see [39].

2.2. Growth and characterization of enamel-like apatite crystals

Crystals were grown in 10% P-Amel with $1 \sim 2$ ppm F in a dual membrane system, which has been used in our previous studies [26,27,29]. It was constructed with a large plastic bottle (volume of 120 ml), a small glass bottle (volume of 2 ml), a cation-selective membrane (CMV™, Asahi Glass Co., Japan), and a dialysis membrane (pore size of 5 nm; Viskase Companies, Inc., Ill., USA). The large bottle was used as a PO_4 solution container (100 ml of 10 mM $NH_4H_2PO_4 + (NH_4)_2HPO_4$; 1:1 molar ratio) and the small glass bottle as a Ca solution container (1.8 ml of 10 mM $Ca(CH_3COO)_2 \cdot H_2O$). Membranes (about 8 mm in diameter) were attached to the Ca solution container by silicone rubber rings and a plastic cap with a hole of about 5 mm diameter. A small reaction space (about 15 μl volume) was constructed between the membranes. NaF solution (1000 ppm) was added to the PO_4 solution to make 1 or 2 ppm F solution. Both Ca and PO_4 solutions were adjusted to pH6.5 by 1 N HCl at 37°C before being used. All chemicals used were reagent grade and all solutions were prepared with deionized and double-distilled water (DDW).

About 1.4 mg of P-Amel weighed by a microbalance was mixed with 13 μl of cold DDW to make 10% amelogenins solution on a cold dish floated on iced water. After protein solution was put into the reaction space between the membranes, the Ca solution container was attached to the PO_4 solution container. The reaction chamber was put into the double-walled 37°C bath. Ca^{2+} and PO_4^{3-} ions diffused into the reaction space from opposite sides, respectively, through the cation selective membrane and the dialysis membrane. The reaction was carried out for 3 days under gentle stirring. After the reaction, the gel fixed on the membrane was rinsed superficially with distilled water, frozen at −80°C, and subsequently lyophilized.

Products on the membrane were identified as they grew, without grinding, by an X-ray diffractometer (XRD) (Rigaku, Nagoya, Japan, RINT 2500, 56 kV, 200 mA, Ni filtered CuKα). Detailed analysis of the deposit on the membrane was performed by means of a