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Altered self-assembly and apatite binding of amelogenin induced by N-terminal proline mutation

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ABSTRACT

Objective: A single Pro-70 to Thr (p.P70T) mutation of amelogenin is known to result in hypomineralised amelogenesis imperfecta (AI). This study aims to test the hypothesis that the given mutation affects the self-assembly of amelogenin molecules and impairs their ability to conduct the growth of apatite crystals.

Design: Recombinant human full-length wild-type (rh174) and p.P70T mutated amelogenins were analysed using dynamic light scattering (DLS), protein quantification assay and atomic force microscopy (AFM) before and after the binding of amelogenins to hydroxyapatite crystals. The crystal growth modulated by both amelogenins in a dynamic titration system was observed using AFM.

Results: As compared to rh174 amelogenin, p.P70T mutant displayed significantly increased sizes of the assemblies, higher binding affinity to apatite, and decreased crystal height.

Conclusion: Pro-70 plays an important structural role in the biologically relevant amelogenin self-assembly. The disturbed regularity of amelogenin nanospheres by this single mutation resulted in an increased binding to apatite and inhibited crystal growth.

1. Introduction

Amelogenesis imperfecta (AI) is a group of genetically transmitted enamel defects.[1,2] Mutational analysis of families with X-linked hypomaturation AI revealed a substitution of adenine for cytosine in exon 6 of the amelogenin gene that results in a proline to threonine change at position 70 (p.P70T) in the protein.[3] The affected enamel exhibits less mineral and a higher protein content as compared to normal enamel.[3–5] Amelogenins are proline-rich proteins that constitute the predominant component in the mineralising enamel matrix. Pro-70 is highly conserved in amelogenin amongst many identified species and is in close proximity to a normal amelogenin cleavage site. Our previous *in vitro* studies verified that the p.P70T substitution caused a delay in the proteolytic cleavage of amelogenin by MMP20.[6] The high conservation and particular location of this proline imply its important functional roles. Despite extensive studies, the mechanism by which the p.P70T mutation of amelogenin distorts the normal enamel formation and hence contributes to the pathogenesis of AI is still unclear. Amelogenin undergoes a spontaneous assembly to form nanospheres that are presumed as the basic building blocks for the initiation and oriented

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elongation of enamel crystals.[7] The assembly is driven by hydrophobic interactions and affected by pH, temperature and protein concentration.[8,9] The tightly associated amelogenin nanospheres interact with apatite crystals, adhering thereto and protecting them from premature fusion.[10] Research has shown that the lack of the hydrophilic C-terminus of amelogenin caused a reduction in the binding affinity to apatite and an interruption of amelogenin self-association.[8,11] During the enamel development, amelogenin proteins are progressively processed and eventually removed from the extracellular space to allow the mineral crystals to grow.[12] The AI-like phenotype observed in amelogenin knockout mice provides further strong evidence for the essential role of amelogenin in the modulation of enamel crystal growth.[13]

We hypothesise that the p.P70T point mutation affects the proper amelogenin–amelogenin and amelogenin–mineral interactions, which results in inhibited enamel crystal growth. These results, together with other basic studies, will provide a deeper insight into the pathological basis of AI, and also advance our understanding of the functions of proline residues in amelogenin. In that respect, the purpose of this paper is dual; on one hand, it serves the purpose of enabling us to grasp a deeper understanding of the bases of AI on the molecular level, whereas on the other hand it aims at elucidating the extent to which a single point mutation, in this case p.P70T, can affect a self-assembly of amelogenin and its interaction with the mineral phase.

2. Materials and methods

2.1. Apatite and protein preparation

Carbonated hydroxyapatite (CHAP) was synthesised as previously described,[14–16] and characterised by X-ray diffraction.[16] Apatite powders were sequentially passed through 30 and 60 μm meshes and only particles with sizes between 30 and 60 μm were collected for the experiments undertaken in this study. The specific surface area of the apatite powder was 74.7 m^2/g.[16]

Recombinant human full-length amelogenin wild-type (rh174) and the mutant with Pro-70 to Thr mutation (p.P70T) were expressed in *E. coli*, purified and identified as described previously.[17,18]

2.2. Dynamic light scattering (DLS) study of assembly

DLS analysis of rh174 amelogenin and p.P70T mutant was carried out using a DynaPro MS/X molecular sizing instrument equipped with a MicroSampler (ProteinSolutions Inc., Charlottesville, VA, USA). Thirty μl of amelogenin (0.5 mg/ml) in 20 mM Tris–HCl buffer was injected into a quartz cell. Tris–HCl was previously adjusted to pH 7.4 at different temperatures to achieve the desired pH value at a given temperature. After inserting the quartz cell into the temperature-controlled chamber, samples were equilibrated for 10 min at 25 °C or 37 °C before DLS data were collected. Approximately 20 DLS measurements were obtained for each sample to provide adequate data replicates.

2.3. Atomic force microscopy (AFM) detection of amelogenin assemblies on apatite

The glass-ceramic used in this study contained rod-shaped fluoroapatite (FAP) crystals embedded in silica matrix.[9,19–21] This glass-ceramic is unique due to the uniaxial alignment of apatite crystals obtained by high-temperature extrusion processing.[19] Prior to the crystal growth experiments, the glass-ceramic substrates were sectioned and polished to expose the predominant (0 0 1) plane of the extruded FAP rods. Each substrate was placed in a prelubricated microcentrifuge tube (Corning, Inc., Corning, NY, USA) containing rh174 at concentration of 0.5 mg/ml (pH 7.4). After incubation with mild shaking for 60 min at 25 °C, the substrates were rinsed with a few drops of deionised water and immediately dried with canned air. The microstructure of proteins immobilised onto the substrates was observed using Nanoscope III AFM (Digital Instruments, Santa Barbara, CA, USA). Images were obtained in dry conditions using the tapping mode with high aspect-ratio Si-tips ($r \sim 5$ nm, $l \sim 125$ μm) (Nanosensors, Neuchatel, Switzerland) operating at approximately 300 kHz.[22]

2.4. Protein assays of amelogenin binding affinity

CHAP (0.5 mg) was thoroughly equilibrated in binding buffer (20 mM Tris–HCl, pH 7.4) in prelubricated tubes. To determine the maximum adsorption amount, different quantities of rh174 and p.P70T amelogenins (20, 40, 60, 80, 100, 120, and 140 μg) were incubated with the equilibrated CHAP for 60 min at room temperature on a shaking incubator. After centrifugation at 5000 × *g* for 5 min, the unbound proteins remaining in the supernatant were quantified by Bradford assay (BioRad, Hercules, CA, USA). The time curves for binding of these two amelogenins were obtained by incubating 150 μg protein with 0.5 mg CHAP and collecting samples at different time points from 0 to 60 min. Experiments were performed in triplicate and statistical differences were calculated by Student's *t* test in Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Both the proteins remaining in supernatant and bound on CHAP were also subjected to SDS-PAGE analysis.

2.5. Amelogenin-guided apatite growth

To study the effects of rH174 and p.P70T on apatite growth, dynamic precipitation experiments were carried out.[23] The polished FAP-glass ceramic substrates were immersed in 0.4 mg/ml protein suspended in 20 mM Tris/HCl (pH 7.40), 150 mM KCl, 0.2% NaN_3 and 2.5 mM KH_2PO_4. Two titrants comprising: (a) 8.2 mM $CaCl_2$, 284 mM KCl and 20 mM Tris/HCl (pH 7.40 ± 0.02), and (b) 5 mM KH_2PO_4, 7 mM KOH, and 20 mM Tris/HCl (pH 7.40 ± 0.02), were subsequently introduced into the reaction system in parallel at the constant rate of 1.2 ml/day at 37 °C, cumulatively, throughout the 7-day period of time. A single substrate was sampled out each day and evaluated for the crystal growth properties using Atomic Force Microscopy (AFM, *Nanoscope III*, *Digital Instruments*). The crystal heights were measured using the section analysis tool in NanoScope software. Heights of twelve randomly chosen peaks in three different 10 μm × 10 μm AFM images were measured and the average values were plotted as a function of