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Dynamic interactions of amelogenin with hydroxyapatite surfaces are dependent on protein phosphorylation and solution pH

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ABSTRACT

Amelogenin, the predominant extracellular matrix protein secreted by ameloblasts, has been shown to be essential for proper tooth enamel formation. In this study, amelogenin adsorption to hydroxyapatite (HAP) surfaces, a prototype for enamel mineral, has been studied using a quartz crystal microbalance (QCM) to interrogate effects of protein phosphorylation and solution pH. Dynamic flow-based experiments were conducted at pH 7.4 and 8.0 using native phosphorylated porcine amelogenin (P173) and recombinant non-phosphorylated porcine amelogenin (rP172). Loading capacities ($\mu mol/m^2$) on HAP surfaces were calculated under all conditions and adsorption affinities (K_{ad}) were calculated when Langmuir isotherm conditions appeared to be met. At pH 8.0, binding characteristics were remarkably similar for the two proteins. However, at pH 7.4 a higher affinity and lower surface loading for the phosphorylated P173 was found compared to any other set of conditions. This suggests that phosphorylated P173 adopts a more extended conformation than non-phosphorylated full-length amelogenin, occupying a larger footprint on the HAP surface. This surface-induced structural difference may help explain why P173 is a more effective inhibitor of spontaneous HAP formation *in vitro* than rP172. Differences in the viscoelastic properties of P173 and rP172 in the adsorbed state were also observed, consistent with noted differences in HAP binding. These collective findings provide new insight into the important role of amelogenin phosphorylation in the mechanism by which amelogenin regulates enamel crystal formation.

1. Introduction

Phosphorylated extracellular proteins, including osteopontin, bone sialoprotein, and dentin matrix protein 1, have been demonstrated to play important roles in mineralization and the regulation of hard tissue formation, in part, due to the presence of multiple phosphorylation sites per protein [1]. Amelogenin, the predominant extracellular enamel matrix protein, is known to be essential for proper tooth enamel formation, but it contains only a single phosphorylation site located within the N-terminal domain [2,3]. Significant progress has been made in advancing the understanding of the structure, function, and role of amelogenin, but details of the effects of key variables, such as post-translational phosphorylation, pH, and truncation and are still debated. The research reported here focuses on the effects of phosphorylation and pH on amelogenin interactions with hydroxyapatite (HAP; $Ca_{10}(PO_4)_6(OH_2)$) surfaces, where HAP is used as a prototype of enamel mineral crystals. Adsorption of amelogenin proteins to HAP was measured under dynamic flow conditions during the first five minutes of protein-surface contact, making this a unique kinetic, rather than commonly-conducted equilibrium [4], analysis of an important protein-mineral interaction. This analysis was made possible through the use of a quartz crystal microbalance (QCM) and focused on both pH 7.4 and 8.0 to show distinct differences between native phosphorylated amelogenin (P173) and recombinant non-phosphorylated amelogenin (rP172) interactions with HAP.

Amelogenin protein structure and function have been recent topics of research interest. It is well accepted that amelogenin and its cleavage products make up over 90% of the extracellular enamel matrix, that amelogenin self-assembles into supramolecular structures in solution in a pH-dependent fashion, and that protein–protein and protein-mineral interactions play a crucial role in mineral regulation [2,5,6]. Full length amelogenin is an

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approximately 20 kDa protein with three significant domains, a hydrophilic N-terminus, a hydrophobic core, and a hydrophilic C-terminus, which are conserved across species [2]. The porcine amelogenin (P173), specifically, has 173 amino acids and undergoes post-translational phosphorylation at the single serine-16 residue [2]. Hence, native forms of amelogenin are phosphorylated, while recombinant versions frequently studied *in vitro* are not. Amelogenin phosphorylation has been shown to greatly influence mineral formation, where phosphorylated amelogenins inhibit HAP formation through the stabilization of amorphous calcium phosphate (ACP) [7–11], but its effect on protein structure and functional mechanism is not yet clear. Although studies using conventional [8] and cryo- TEM [9] have shown many similarities between phosphorylated and non-phosphorylated amelogenins with respect to structure and self-assembly, subtle differences were noted that might explain the profound effect phosphorylation has on mineralization [7,8]. Small-angle X-ray scattering (SAXS) studies using the leucine-rich amelogenin peptide (LRAP) from pig, a 56-amino-acid alternative splice product of the amelogenin gene composed of the first 33 N-terminal and the last 23 C-terminal amino acids of full-length amelogenin, have also shown that phosphorylation induces significant changes in protein folding that may further affect protein-mineral interactions [10]. A key feature of amelogenin proteins is their lack of monomeric stability under physiological conditions and their propensity to form nano-sized particle aggregates (so-called nanospheres) [8,12–16]. These nanospheres, that appear to be comprised of a dense protein core with a loose outer shell structure, have been shown to have the capacity to form higher-order assemblies in solution in a pH-dependent fashion. Aggregates appear smaller, as 15–20 nm diameter nanospheres, at approximately pH 8 and much larger as pH decreases to physiological conditions, where proteins ultimately enter a gel-like phase where chain-like assemblies of the 'nano-sphere' subunits are observed [8,12]. Additional studies using SAXS showed that these amelogenin building blocks actually adopt a non-spherical anisotropic oblate shape [13], which helps to explain the capacity of full-length amelogenin to form linear arrays.

Solution NMR and FT-IR studies have provided additional insight, showing that the monomeric protein is intrinsically disordered but that it adopts different structures when interacting with molecular moieties [17,18]. Methods of solid-state NMR have opened the door to challenging but high resolution structural biology studies [19] of amelogenin in a mineral-bound state. Shaw and colleagues have recently demonstrated that mouse amelogenin undergoes a random coil to beta-sheet transition upon mineral binding [20]. that surface-induced structural changes occur for both phosphorylated and non-phosphorylated forms of LRAP [21], and that molecular level interactions may change with phosphorylation state leading to macroscopic differences in adsorption or mineralization [22]. Additional analytical tools have been used to study amelogenin structure and function in solution and on surfaces with some success, including FT-IR [18], dynamic light scattering (DLS) [8,12], electron microscopy [9,13,14], atomic force microscopy [15,16], SAXS [2,13], and end-point solution analyses [4]. Significant drawbacks of these approaches include the inherent structural challenges of working with a disordered protein, the large amount of sample required per experiment, the time required for data collection and analysis, and the lack of real-time, dynamic results with high temporal resolution. Studies such as these provide a high-resolution end-point analysis of bound protein structure, but need to be complemented by dynamic and kinetic measurements that demonstrate important aspects of protein behavior.

QCM is a technique that has been widely used as a biosensor to study protein-surface interactions in real-time [23,24], and is capable of providing high sensitivity, real-time analysis of protein adsorption *in situ* based on mass adsorption and changes in local viscoelasticity, recently reviewed by Reviakine, et al. [25], and long studied by others [26–30]. The role that amelogenin plays in mineral formation and stabilization of precursor phases by necessity relates to adsorption, affinity, and surface coverage, but may also be affected by kinetic factors. QCM allows for such useful and complementary measurements of adsorption, including initial kinetic rates, surface loading capacity, affinity constants, and surface rearrangement or conformational changes. Importantly, the micro-flow analysis format of these experiments is efficient in time and sample consumption. The summary of these results can provide important insight into the interaction between proteins and surfaces, as has been demonstrated in the QCM analysis of enamel matrix and salivary proteins on a variety of surfaces [31–36]. While QCM does not provide detailed structural information on a protein, the functional and dynamic aspects of adsorption and surface coverage lend to support detailed structural studies and connect structure to function, which is needed to better understand the role of single-point phosphorylation in amelogenin proteins.

2. Materials and methods

2.1. Chemicals

Hydroxyapatite nanopowder (<200 nm and >97% purity) and tris hydrochloride were purchased from Sigma-Aldrich. Ethanol (95%) was purchased from Carolina Biological. Native (P173) and recombinant (rP172) full-length amelogenins were provided by Drs. James Simmer and Yasuo Yamakoshi, prepared as previously described [37,38] and stored at The Forsyth Institute. The rP172 lacks a sole phosphorylated site (S-16) and an N-terminal methionine found in the native P173. All other chemicals were reagent grade and used as received.

2.2. Quartz crystal microbalance

A quartz crystal microbalance (QCM) sensor was used in order to analyze protein adsorption to HAP surfaces in real time with high sensitivity. The QCM (Stanford Research Systems, QCM 200) was operated in an equivalent circuit mode [26,28,39] and measurements of resonant frequency (Δf, Hz) and loading resistance (ΔR, Ω) were made. In solution, Δf responds to both mass adsorption (Δm) and to density and viscosity ($\Delta \rho h$) changes at the surface-solution interface, while ΔR responds only to density and viscosity effects. The instrument was calibrated for changes in ΔR using sucrose solutions that alter the solution density and viscosity, but do not adsorb to the gold electrode. By taking both Δf and ΔR into consideration through this calibration, accurate mass measurements are made. This approach has been described previously [40,41]. Changes in ΔR can also be used to gain information about local density and viscosity differences that arise from changes (*e.g.*, protein rearrangement due to changes in protein–protein and/or protein-mineral interactions) in adsorbed protein layers [25,28]. Previous work has demonstrated that ΔR measurements are theoretically and practically equivalent to dissipation measurements (ΔD) made with QCM-D instruments [25,42], which indicate energy variation in the system by changes in viscoelasticity. No change in ΔR or ΔD would indicate the adhesion of a rigid layer. Experiments used 1 in. diameter Ti/Au polished 5 MHz quartz crystals (Stanford Research Systems) and 100 μL axial flow cell. Solutions were controlled with a peristaltic pump (Masterflex C/L) and Tygon tubing (Cole – Parmer 0.020 in ID 0.092 OD) with flow rates of 25 μL/min for dynamic flow experiments and 125 μL/min for initial injection in static experiments. Fresh tubing was used for each experiment to limit contamination and flow rate deviations. All experiments were carried out at room temperature.