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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 ($\mu\text{mol}/\text{m}^2$) 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.

Graphical Abstract

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Supporting Information.

AFM of HAP spin-coated onto a QCM crystal; Kinetic rates for the first two minutes of binding in dynamic experiments; Langmuir isotherm plots for dynamic QCM experiments; Complete binding curves at additional concentrations for QCM dynamic experiments; Static binding plots and Langmuir isotherm plots for static QCM experiments.

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Keywords

amelogenin; phosphorylation; hydroxyapatite; quartz crystal microbalance; adsorption

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; $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH}_2)$) 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 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.

MATERIALS AND METHODS

Chemicals

Hydroxyapatite nanopowder (<200nm 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.

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 (f , Hz) and loading resistance (R , Ω) were made. In solution, f responds to both mass adsorption (m) and to density and viscosity ($\rho\eta$) changes at the surface-solution interface, while R responds only to density and viscosity effects. The instrument was calibrated for changes in R using sucrose solutions that alter the solution density and viscosity, but do not adsorb to the gold electrode. By taking both f and R into consideration through this calibration, accurate mass measurements are made. This approach has been described previously.[40,41] Changes in R 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 R measurements are theoretically and practically equivalent to dissipation measurements (D) made with QCM-D instruments,[25,42] which indicate energy variation in the system by changes in viscoelasticity. No change in R or D 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 μ L 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 μ L/min for dynamic flow experiments and 125 μ L/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.

An HAP surface was prepared on the QCM gold electrode using a suspension of 5 mg/ml solid HAP in ethanol. The suspension was sonicated (Sonicwise Ultrasonics) for 20 minutes then added drop-wise to the QCM crystal on a home-built spin-coater, rotating at 1140 rpm. A total of 200 μ l of the suspension were added to the center of the crystal, allowing drying time between each drop. Additional time was allowed for drying at room temperature prior to use in the QCM. Surfaces were reused after rinsing in three washes of 6 M HCl to dissolve the mineral layer, followed by DI water and ethanol rinses, and drying with airflow. A new HAP coating was used for each protein binding experiment. The stability of the mineral layer was also examined under these experimental conditions, as described below.

Hydroxyapatite Layer Characterization

HAP was characterized using optical microscopy, SEM, and FT-IR. Electron microscopy was completed using a Zeiss EVO LS10 microscope operated at 2.0 kV. FT-IR spectra were combined from 64 scans on a Thermo Nicolet Avatar 370 with a diamond crystal Smart iTR attachment. QCM crystals were prepared as above. Surface roughness of spin-coated HAP was determined by AFM (Bruker Nanoscope III multimode, Santa Barbara, CA) in contact mode with a SiN₄ cantilever. Surface area measurements were obtained using the instrument's accompanying analysis software.

Amelogenin Protein Preparation

Prepared frozen protein stock solutions (5 mg/ml in DI water) were received from The Forsyth Institute. Stock solutions were then stored at 4°C and used within two weeks of being received. Dilutions were made in 20 mM tris buffer at pH 7.4 or pH 8.0 to yield the desired protein concentration. Prior to use, complete dissolution of protein was confirmed using dynamic light scattering (DLS), as previously described.[8] Stock solutions in DI water exhibited mean hydrodynamic radii values (R_H) between 1 – 2 nm, consistent with the presence of monomers or dimers.

Amelogenin Binding

The QCM axial flow cell was filled with 20 mM tris buffer, at pH 7.4 or 8.0, using a peristaltic pump. Electrode capacitance was adjusted to zero in the presence of buffer prior to each experiment. The electrode surface was equilibrated in buffer to achieve a stable baseline prior to injecting protein solution at a constant rate of 25 μ L/min for 5.0 min. Buffer was then introduced for a minimum of 10 min to wash unbound protein from the flow cell. Additional methods for static binding experiments can be found in Supporting Information, S5.

Isotherm Analysis

The Langmuir isotherm was used to determine affinity constants (K_{ad} , M⁻¹) and surface loading at saturation (N) when appropriate,[4,39,40,43]

$$\frac{C}{Q} = \frac{1}{N}C + \frac{1}{K_{ad}N} \quad (1)$$

where, C is the initial concentration of protein in solution in mol/L, Q is the amount of material bound in g/m^2 or $\mu\text{mol/m}^2$, N is the maximum surface loading in g/m^2 or $\mu\text{mol/m}^2$, and K_{ad} is the affinity constant in M^{-1} . These constants were obtained from a least-squares fitting of the linearized form of the Langmuir isotherm (*i.e.*, a plot of C/Q against C), according to eq. 1. Error for these values is given as a standard deviation propagated from the regression analysis of the linear Langmuir model fit, where a minimum of six data points ($n=6$) were used to calculate each value. The appropriateness of this model to describe the data was assessed based on an acceptable fit.

RESULTS

Surface Preparation and Characterization

Complete coverage of the gold QCM electrode with HAP is crucial to gaining specificity for protein adsorption. Hydroxyapatite nano-powder characterization from the manufacturer confirmed the structure by FT-IR spectroscopy, complexometric titration, atomic emission spectroscopy, and TEM analysis.[44] Additional analysis was performed after dispersing the solid onto the QCM surface by spin-coating. Figure 1A shows an optical microscopy image of the coated QCM surface, demonstrating uniformity of the surface layer. The HAP can be seen as a white coating on what would otherwise be a reflective gold electrode. Figure 1B shows an SEM image of the coated QCM surface, demonstrating that the gold is completely and uniformly covered at the micron scale. Figure 1C is the FT-IR spectrum of the HAP showing characteristically sharp peaks at 1061, 962, 604, and 575 cm^{-1} . [45,46] The HAP rigidly adsorbed to the gold electrode and was stable in tris buffer under static and flowing conditions, as confirmed by a steady QCM baseline prior to protein injection in Figure 2. On the bulk scale, this material demonstrates no specific crystal orientation, which is desirable to achieve a randomly arranged surface that is relatively homogenous. AFM measurements determined the surface area of HAP on the sensitive area of the QCM crystal to be $7.49 \times 10^{-5}\text{ m}^2$, which is 2.19 times greater than the area of the gold sensor. Error associated with this measurement would affect derived units (*i.e.*, $\text{nm}^2/\text{protein}$ or $\mu\text{mol/m}^2$) equally, possibly changing the magnitude of the derived value, but not the relationship between the values. AFM images can be found in Supporting Information, S1.

Dynamic Flow QCM

Proteins rP172 and P173 (Figure 2) were found to bind to HAP with dependence on both solution protein concentration and solution pH. When the protein reaches the sensor surface, measured mass increases consistently for the duration of the 5 min injection. Mass binding ceases at the end of the 5 min injection when buffer washes unbound protein out of the flow cell and promotes some protein desorption due to a shift in solution equilibrium. At pH 8.0 (Figure 2B), binding characteristics are remarkably similar for the two proteins, including minor desorption during the buffer wash. Kinetic rates ($\mu\text{g/min}$) for the initial 2 min of binding increase with increasing protein concentration, as anticipated, and the correlation between rate and protein concentration is statistically equivalent for the two proteins (Supporting Information, S2). Peak mass adsorption at each concentration, Figure 3, is relatively low (less than 0.012 g/m^2) at pH 8.0 with no indication of surface saturation in this concentration range. The proteins are similar in calculated loading capacities at a $15\text{ }\mu\text{M}$

concentration ($N_{15\mu\text{M}}$) of 0.58 and 0.59 $\mu\text{mol}/\text{m}^2$ for rP172 and P173, respectively (Table 1). $N_{15\mu\text{M}}$ is used for comparison because no Langmuir fit was possible at pH 8.0.

In contrast, initial binding rate and total mass adsorption increase significantly for both proteins at pH 7.4 compared to that found at pH 8.0. Kinetic rates in the first 2 min of binding are statistically equivalent for rP172 and P173 at pH 7.4, as they were at pH 8.0 (Supporting Information, S2), but mass adsorption is higher for rP172 and lower for P173 (Figure 3). Both proteins show surface saturation at increasing protein concentration at pH 7.4. Plotting adsorption data with a linear Langmuir model (Supporting Information, S3) allows for the calculation of equilibrium adsorption constants, K_{ad} , given in Table 1. Protein P173 was found to have a significantly higher K_{ad} , indicating a higher affinity for HAP. P173 saturation of the HAP surface to form a monolayer was also found to take place at a lower protein concentration, in comparison to rP172.

Isotherm modeling allows for a calculation of the theoretical maximum mass adsorbed (N) when saturation to a monolayer is reached. As seen in Table 1, P173 saturates at approximately half the mass loading of rP172 at pH 7.4, at $0.021 \pm 0.004 \text{ g}/\text{m}^2$ and $0.04 \pm 0.02 \text{ g}/\text{m}^2$, respectively. Expressed in mole amount, N is $1.0 \pm 0.2 \mu\text{mol}/\text{m}^2$ and $2.1 \pm 0.9 \mu\text{mol}/\text{m}^2$ for P173 and rP172, respectively. Based on the surface area of HAP for adsorption, determined by AFM, P173 occupies a larger surface footprint of $1.6 \pm 0.3 \text{ nm}^2$ compared to rP172 of $0.8 \pm 0.3 \text{ nm}^2$.

The data represented in Figure 2 and Figure 3 can be replotted to show the change in R with increasing mass loading, seen in Figure 4. In all cases, only minor differences in R/m profiles are noted when comparing high (15 μM) and low (2.5–3.75 μM) concentrations, indicating the reproducibility of these experiments and that any structural change is not dependent on solution concentration. On the other hand, significant changes in R are observed with increasing surface mass loading. When considering amelogenin rP172 (Figure 4), R/m profiles are very similar at both pH values in low mass loading, up to $2 \times 10^{-3} \text{ g}/\text{m}^2$, with R near zero. With increasing mass loading, R becomes significantly negative at both pH values, though with different R/m slopes. When considering amelogenin P173, the differences at each pH value are immediately more distinct. At low mass loading, R is positive at pH 8.0 and negative at pH 7.4. As mass loading increases, R becomes negative in all cases, but P173 at pH 7.4 demonstrates the largest viscoelastic change with the lowest mass loading. For example, at pH 7.4, P173 reaches a R of -40Ω at $0.018 \text{ g}/\text{m}^2$, while rP172 requires a larger mass of $0.027 \text{ g}/\text{m}^2$ to reach the same R .

Static QCM

For comparison, additional amelogenin binding experiments were conducted under static, no-flow conditions over a period of 120 min to explore any changes that occur over a longer timeframe (Supporting Information, S5). It is in these experiments that amelogenin proteins reach a stable, equilibrium steady state after approximately 20–30 min with only a fraction (~2–3%) of the total protein in solution binding to the HAP surface. Both proteins show a general linear response with signs of surface saturation at pH 7.4 at high concentrations.

DISCUSSION

The importance of amelogenin solution structure,[12–14,17,47] surface structure,[15,16,20–22,48] adsorption properties,[4,49] and role in nucleation and organization of calcium phosphate mineral[5,7–9,50–52] has been well documented. Particular attention has been paid to solution pH and temperature, truncation, and the phosphorylation state of the protein, since these factors have been shown to have a direct impact on amelogenin self-assembly, protein-mineral interaction, and the regulation of mineralization processes.[7–9,12,13,20–22,50] Work presented here demonstrates distinct differences that arise in the dynamic adsorption of amelogenin to pre-formed HAP, based on solution pH and protein phosphorylation, measured by QCM. It is important to note that kinetic binding experiments such as these do not necessarily allow for an equilibrium state to be reached between the protein and the surface in the typical, short 1–10 min time frame, though may provide information that is complimentary and unique compared to other studies. Similar protein-surface interactions have typically been studied on much longer time scales (2–24 hr) [4,33,51] to reach full equilibration. Long time-scale experiments were also conducted with the QCM for comparison by arresting flow in a static experiment.

Prior to adsorption experiments, it was imperative to ensure a stable calcium phosphate layer with complete coverage of the QCM electrode and the characteristics of crystalline HAP. Optical and electron microscopy in Figure 1 demonstrate the extensive coverage of the HAP layer and FT-IR spectroscopy demonstrates that the mineral has similar characteristics to a NIST standard HAP. Spin-coating HAP onto the gold QCM surface proved to be a reliable method of deposition and HAP appeared to have a natural adherence to gold, forming a stable surface. The mass or thickness of HAP deposited, in this case, is irrelevant so long as the surface is completely covered and the QCM is not overloaded. Stability of the HAP layer can be observed in the first minutes displayed in Figure 2, where a stable baseline is recorded.

The first observation made when comparing adsorption of the two amelogenin proteins is that at pH 8.0 they behave with remarkable similarity with regard to loading capacity, footprint, and apparent mechanism of adsorption to HAP. At this pH, the protein is known to form nanometer-sized aggregates in solution.[12,14,47,50,53] This corresponds to a relatively low average loading capacity ($N_{15\mu\text{M}}$) of $0.56 \pm 0.04 \mu\text{mol}/\text{m}^2$ for both proteins and possible lateral surface interactions or aggregation, not matching Langmuir isotherm trends. Dynamic and static experiments appear to agree at pH 8.0, as loading capacities are similar, suggesting that equilibrium is reached in both experiments. Previous *static* experiments by Bouropoulos, *et al.*[4] studied recombinant, non-phosphorylated mouse amelogenin rM179 adsorption to HAP at pH 8.0 and found surface saturation with Langmuir isotherm agreement, in contrast to our findings. These experiments were conducted in a similar concentration range (0.1–27.5 μM compared to 0.5–15 μM) and found similar surface loading capacities at 15 μM solution concentration (0.609 $\mu\text{mol}/\text{m}^2$ compared to 0.51–0.58 $\mu\text{mol}/\text{m}^2$) to this work, but their experiments were equilibrated in solution with HAP for 20 h. This may indicate that the non-phosphorylated mouse amelogenins require longer times to reach equilibrium with mineral surfaces and that dynamic experiments may

provide unique adsorption information. Bouropoulos, *et al.*[4] did not study phosphorylated amelogenin or physiological pH conditions for comparison.

Significant differences arise in QCM binding data when the pH is lowered to pH 7.4. At this pH, both proteins show higher loading capacity ($N_{15\mu M}$) in dynamic experiments of 0.89–1.34 $\mu\text{mol}/\text{m}^2$ compared to 0.51–0.58 $\mu\text{mol}/\text{m}^2$ at pH 8.0. We also observe apparent agreement with Langmuir isotherm models indicating a homogenous surface, no protein-protein interactions, and binding saturation to a monolayer (Figure 3), not seen in pH 8.0 experiments.[40,43] The variation from pH 8.0 to pH 7.4 clearly has a significant impact on the structure and function of these proteins. This is in agreement with previous DLS, SAXS, and TEM work.[8,9,12–14,54] At the higher pH value, full length amelogenin forms stable nanospheres in solution. As the pH approaches physiological conditions, larger aggregates begin to form. This increase in solution aggregation might appear to support our QCM findings that a higher loading capacity is achieved at lower pH values, but an agreement with the Langmuir isotherm at lower pH and not at higher pH argues against multi-layer aggregation. The interaction at the surface may be different than what is observed in solution, as seen with salivary proteins.[55,56] In agreement with this finding, Tarasevich *et al.*[49] recently suggested a “shedding” mechanism where solution nanospheres of LRAP disassemble to monomers or dimers at pH 7.4 at an HAP surface. This pH effect may also relate to protein surface charge, but surface charge distribution is not known for these proteins. Isoelectric point (pI) has been previously studied theoretically and experimentally[57] for various amelogenin proteins. Experimental and theoretical pIs vary depending on the protein and the software used to obtain theoretical estimates. Using software that takes phosphorylation into consideration,[58] the pI was calculated to be 6.35 for phosphorylated P173 and 6.55 for non-phosphorylated rP172. The lower pI for P173 suggests an overall more negative charge than rP172 and the difference between the two proteins would be more pronounced at pH 7.4 than 8.0, which also supports the observed differences in binding.

It is also apparent that at pH 7.4, significant differences arise between protein phosphorylation states that were not seen at pH 8.0. At pH 7.4, P173 binds to HAP with a higher affinity (K_{ad}) and correspondingly lower loading capacity (N) compared to rP172, consistent with previous reports that phosphorylated LRAP binds to HAP to a lower extent than non-phosphorylated LRAP.[22] If surface saturation is held to a monolayer and protein-protein aggregation is low, fitting with the assumptions of the Langmuir isotherm, then in order to accommodate the significantly lower loading capacity, the phosphorylated amelogenin P173 protein may adopt an extended conformation with a larger footprint or rP172 may contract to occupy a smaller footprint. At the theoretical maximum loading capacity (N) in Table 1, the protein footprint would correspond to $1.6 \pm 0.3 \text{ nm}^2$ for P173 compared $0.8 \pm 0.3 \text{ nm}^2$ for rP172. Consistent with this finding, prior DLS results have shown that P173 and rP172 form nanospheres of the same size at higher pH values over a wide range of ionic strength (IS) and form the same higher-order aggregate chain-like structures, but at *different* lower pH values that varied somewhat with IS.[8] Complementary NMR studies with mouse amelogenin and LRAP also support these findings by observing structural switching upon binding and as a function of phosphorylation.[20–22]

The assessment of adsorption kinetics, made possible with dynamic QCM measurements, provides additional support of surface rearrangement and structural switching (Supporting Information, S2). Initial rates ($0.025 \pm 0.002 \mu\text{g}/\text{min}$, $t = 0\text{--}2 \text{ min}$) of adsorption at pH 7.4 are statistically equivalent regardless of the phosphorylation state, but the final loading capacity is significantly different (Table 1). This indicates that a difference in binding occurs in the latter half of the 5 min injection. One hypothesis is that adsorption of amelogenin to the HAP surface is initially non-specific and compulsory, but is followed by a period of equilibration, rearrangement, and structural switching on the surface that results in saturation at higher protein concentrations. This observation supports previous findings that these proteins have similar behavior or structure in solution, but undergo structural changes upon adsorption, as observed using FT-IR for rP172.[18]

Static QCM experiments (Supporting Information, S5) were conducted to make comparison with kinetic adsorption results. Importantly, static experiments demonstrate that both amelogenin proteins reach stable adsorption states and do not continue to aggregate or bind non-specifically over a 2 h period on the HAP surface, potentially adding to the validity of kinetic experiments. Adsorption loading capacities ($N_{15\mu\text{M}}$) are shown for comparison in Table 1 and in Supporting Information, S5.

A significant advantage of the QCM, relative to traditional approaches to elucidate the mechanism of protein binding to surfaces, is in its ability to interrogate viscoelastic properties in addition to mass loading. When rigid materials adhere to the QCM gold electrode, the acoustic wave generated by the resonant quartz propagates with minimal energy loss through the solid material then penetrates into the contact media as an evanescent wave. Biomolecules are not considered rigid, but have viscoelastic properties that interact with the evanescent acoustic wave. Rigorously solving mathematical models to calculate energy loss to a biomolecule is not trivial, but differences in viscoelastic properties are measured as loading resistance, R , and can be used *qualitatively* to differentiate structural characteristics, as described by Reviakine *et al.*[25] and others.[26–29,59] Small changes in R with increasing mass (R near zero) would indicate mass loading with only minor changes in viscoelasticity, or loading of relatively rigid mass. Large changes in R , positive or negative, would indicate shifts in the viscoelastic properties of the adsorbing mass and non-rigid mass loading. In general, the difference between the surface viscoelastic properties of rP172 at pH 7.4 and 8.0 is measurable, but not drastic. When considering amelogenin P173, on the other hand, the differences are more distinct (Figure 4), with P173 reaching a large negative R value at relatively low mass loading at pH 7.4. This indicates that there is a more distinct difference between the adsorbed protein layers in two situations: 1) when comparing amelogenin P173 at two pH values and 2) when comparing rP172 to P173 at pH 7.4. The large R shift at low mass loading seen with P173 suggests that each P173 molecule is affecting a large portion of the HAP surface. This supports our conclusion that P173 undergoes a conformational change at the HAP surface to adopt a larger footprint. The hypothesis of P173 elongation on the HAP surface, presented here, is consistent and well-supported by our present findings, although other possible mechanisms to explain lower P173 saturation binding cannot be ruled out at this time.

CONCLUSION

We conclude from these data that at pH 8.0 both rP172 and P173 have similar interactions in solution and at the HAP surface. At pH 7.4 the phosphorylated P173 adopts a more extended conformation upon adsorption resulting in a higher affinity and larger footprint. The ability of P173 to bind with higher affinity, cover an HAP surface with fewer protein molecules, and more rapidly equilibrate with mineral surfaces, as observed in the present study, suggests that native amelogenin phosphorylation may markedly affect its capacity to regulate enamel mineral formation by better stabilizing mineral phases.[8,11] Our present findings demonstrate how a single phosphate group can alter dynamic interactions of a protein with an HAP surface and potentially play a critical role in regulation of dental enamel formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Dynamic QCM measurements provide unique kinetic surface adsorption information
- QCM loading resistance shows differences in protein-surface viscoelastic properties
- Amelogenin with phosphorylation has higher affinity and larger footprint at pH 7.4
- A single phosphate group alters dynamic amelogenin-hydroxyapatite interactions

Figure 1.

Characterization of HAP on the QCM crystal surface. A) Optical microscopy, showing uniform distribution of HAP particles on the QCM gold electrode. B) SEM microscopy, showing complete coverage of the QCM gold electrodes and uniform distribution of HAP particles. C) FT-IR of the QCM surface shows peaks at 1061, 962, 604, and 575 cm^{-1} , indicative of HAP. FT-IR of a NIST standard HAP is shown for comparison.

Figure 2. Representative QCM binding curves for amelogenin protein adsorption on HAP surfaces at 25 µL/min flow for a 5 min injection. A) Binding at pH 7.4; B) Binding at pH 8.0. Binding curves at all concentrations can be found in Supporting Information, S3.

Figure 3. QCM data for amelogenin protein adsorption on HAP surfaces at 25 $\mu\text{L}/\text{min}$ flow for a 5 min injection, shown as mass adsorption (left axis) and corresponding mole adsorption (right axis). Increasing solution concentration causes an increase in final adsorbed amount. At pH 7.4, both proteins exhibit binding saturation at high concentrations. At pH 8.0, both proteins exhibit a linear relationship between concentration and adsorption. A) Binding at pH 7.4; B) Binding at pH 8.0.

Figure 4.

Representative resistance plots of amelogenin protein adsorption on HAP surfaces at 25 $\mu\text{L}/\text{min}$ flow for a 5 min injection. Changes in resistance with mass adsorption indicate changes in local density and viscosity or changes at the HAP surface. **A)** Binding at pH 7.4; **B)** Showing a smaller resistance and mass range of (A); **C)** Binding at pH 8.0; **D)** Showing a smaller resistance and mass range of (C).

Table 1

Results from 5 min flow injection experiments show a higher affinity and lower maximum mass adsorption for P173 compared to rP172. Amelogenin rP172 binds with a higher loading capacity than P173. Both proteins exhibit unique behavior at pH 7.4, but similar behavior at pH 8.0. Results from 120 min static experiments show similarities with dynamic experiments at pH 8.0, but lower loading capacity at pH 7.4.

	rP172		P173	
	pH 7.4	pH 8.0	pH 7.4	pH 8.0
Dynamic Experiments³				
$K_{ad} (M^{-1}) \times 10^5$	0.8 ± 0.3	-- ¹	5 ± 1	-- ¹
$N (g/m^2)$	0.04 ± 0.02	-- ¹	0.021 ± 0.004	-- ¹
$N (\mu mol/m^2)$	2.1 ± 0.9	-- ¹	1.0 ± 0.2	-- ¹
$^2N_{15\mu M} (\mu mol/m^2)$	1.34	0.58	0.89	0.59
Static Experiments				
$^2N_{15\mu M} (\mu mol/m^2)$	0.71	0.51	0.54	0.57

¹Not calculated due to poor fit with Langmuir isotherm model.

²Maximum adsorption is given at 15 μM concentration in order to make comparisons with binding that did not fit a Langmuir isotherm model.

³Error is given as a standard deviation propagated from the regression analysis of the linear Langmuir model fit.