Published in final edited form as:

J Struct Biol. 2010 March ; 169(3): 266–276. doi:10.1016/j.jsb.2009.10.007.

The leucine rich amelogenin protein (LRAP) adsorbs as monomers or dimers onto surfaces

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Abstract

Amelogenin is believed to be involved in controlling the formation of the highly anisotropic and ordered hydroxyapatite crystallites that form enamel. The adsorption behavior of amelogenin proteins onto substrates is very important because protein–surface interactions are critical to its function. We have previously used LRAP, a splice variant of amelogenin, as a model protein for the full-length amelogenin in solid-state NMR and neutron reflectivity studies at interfaces. In this work, we examined the adsorption behavior of LRAP in greater detail using model self-assembled monolayers containing COOH, CH₃, and NH₂ end groups as substrates. Dynamic light scattering (DLS) experiments indicated that LRAP in phosphate buffered saline and solutions containing low concentrations of calcium and phosphate consisted of aggregates of nanospheres. Null ellipsometry and atomic force microscopy (AFM) were used to study protein adsorption amounts and quaternary structures on the surfaces. Relatively high amounts of adsorption occurred onto the CH₃ and NH₂ surfaces from both buffer solutions. Adsorption was also promoted onto COOH surfaces only when calcium was present in the solutions suggesting an interaction that involves calcium bridging with the negatively charged C-terminus. The ellipsometry and AFM studies revealed that LRAP adsorbed onto the surfaces as small subnanosphere-sized structures such as monomers or dimers. We propose that the monomers/dimers were present in solution even though they were not detected by DLS or that they adsorbed onto the surfaces by disassembling or “shedding” from the nanospheres that are present in solution. This work reveals the importance of small subnanosphere-sized structures of LRAP at interfaces.

Keywords

Amelogenin; LRAP; Adsorption; Quaternary structure

1. Introduction

Amelogenin proteins are believed to be involved in the formation of the mineralized calcium phosphate enamel structures found in teeth (Fincham et al., 1999). Enamel consists of unusually high aspect ratio carbonated hydroxyapatite crystals, which assemble into bundles that are in turn assembled into woven structures. The 90% mineral content, high aspect ratio crystals, and complex self-assembled structures are believed to contribute to the exceptional hardness properties of teeth. The function of amelogenin is not conclusively known, but roles in nucleation (Tarasevich et al., 2007; Wang et al., 2007), growth (Beniash et al., 2005; Iijima et al., 2001), assembly (Moradian-Oldak et al., 1998c), and spacing of crystallites (Fincham et al., 1995) have been proposed. Several in vivo studies using antisense mice (Diekwisch et al., 1993), knock-out mice (Gibson et al., 2001), transgenic mice (Paine et al.,

2000), and hammerhead ribozymes (Lyngstadaas et al., 1995) have found that the absence or reduction of amelogenin greatly affected the degree of mineralization and organization of crystallites within enamel. The proposed functions for amelogenin involve the interactions of the protein with mineral surfaces, indicating that the adsorption behavior of amelogenin has great importance and is critical to its function.

Full-length amelogenin is a ~20 kDa protein that contains a hydrophobic central region and charged residues found in the C-terminal domain and near the N-terminus. Amelogenin is unique in that it can self-assemble in solution to form supramolecular structures called “nanospheres,” assemblies of protein monomers that are typically 20–60 nm in diameter (Moradian-Oldak et al., 1998b, 1994). Nanospheres have been observed in vivo, within growing enamel (Fincham et al., 1995; Moradian-Oldak and Goldberg, 2005). In addition to full-length amelogenin, the leucine rich amelogenin protein, LRAP, is also present during enamel mineralization (Fincham et al., 1982). This protein is a 59 residue alternative splice variant, translated from exons 2, 3, 5, 6d, and 7 of amelogenin mRNA (Gibson et al., 1991). LRAP consists of the highly conserved N- and C-terminal regions of amelogenin, without the large central hydrophobic residues, as shown in Table 1. Like amelogenin, it is believed that LRAP may have a similar nanosphere quaternary structure since nanospheres have been observed adsorbed onto hydroxyapatite (Iijima et al., 2001) and fluoroapatite surfaces (Habelitz et al., 2006).

Although there has been less work done to try to understand the role of LRAP, a range of functions have been proposed including crystal growth inhibitor (Habelitz et al., 2006; Moradian-Oldak et al., 1998a), enamel formation promotor (Gibson et al., 2008; Ravindranath et al., 2007), and cell signaling molecule (Boabaid et al., 2004; Veis, 2003; Warotayanont et al., 2008). Mouse molar explant studies by Ravindranath et al. showed that enamel crystals were thicker in amelogenin knock-out mice that were exposed to LRAP (Ravindranath et al., 2007). Gibson et al. showed that enamel crystals of amelogenin knock-out mice that were mated with transgenic LRAP mice recovered some of their enamel crystal organization (Gibson et al., 2008). Other studies have shown that the expression of LRAP in amelogenin knock-out animals did not completely restore the normal enamel phenotype (Chen et al., 2003). It should be noted, however, that even amelogenin did not completely restore complete enamel thickness in amelogenin knock-out mice suggesting that other proteins including the LRAP splice variant may aid in enamel formation (Li et al., 2008). Other studies have suggested that LRAP may have quite a different role than enamel mineralization – functioning as a cell signaling molecule promoting epithelial–mesenchymal interactions. LRAP has been found to induce osteogenesis in various cell types including rat muscle fibroblasts (Veis et al., 2000), mouse cementoblasts (Boabaid et al., 2004), and mouse embryonic stem cells (Warotayanont et al., 2008).

Although the biological role of LRAP is currently under debate and may or may not involve enamel formation, LRAP has been found to be a very useful protein to act as a model for amelogenin in solid-state NMR (Shaw and Ferris, 2008; Shaw et al., 2004a, 2008) and neutron reflectivity studies (Shaw et al., 2004b). Although missing the central hydrophobic region, the N-terminal and C-terminal regions believed to be crucial for nanosphere assembly and adsorption interactions with hydroxyapatite are conserved in LRAP. Because LRAP is a relatively small protein, it can be synthesized using solid phase protein synthesis techniques, allowing the incorporation of isotopic labels such as ^{13}C , ^{15}N , and deuterium into specific residues of the protein. Full-length amelogenin is too large to be synthesized using solid-phase synthesis and is therefore synthesized using recombinant techniques, preventing the incorporation of site-specific labels. Site-specific labeling of LRAP allows the determination of secondary structure in specific regions of LRAP as well as the intersite spacing between labeled residues in the protein and ^{31}P surface sites in hydroxyapatite. Our

goals, therefore, are to use LRAP as a model protein for amelogenin. This approach has been very valuable, providing a highly detailed, molecular level understanding of the structure of LRAP on hydroxyapatite and other surfaces. The SSNMR studies have shown for the first time that the C-terminal domain of LRAP interacts with the surface and lies down flat at the surface from residues 42 to 58 and has a random coil secondary structure (Shaw and Ferris, 2008; Shaw et al., 2008). Since the C-terminal domain is conserved between LRAP and amelogenin, we believe that the structure of amelogenin in the near surface region is similar to the surface structure we have determined for LRAP.

We report studies of the adsorption of LRAP onto self-assembled monolayers (SAMs) on gold containing NH_2 , CH_3 , and COOH functionality. The SAM surfaces are good model systems with highly controlled structures and chemistries and have been useful for studying fundamental aspects of protein adsorption (Lestelius et al., 1997; Prime and Whitesides, 1993). Our goals are to develop a better understanding of the adsorption behavior of LRAP onto surfaces using ellipsometry to determine adsorption isotherms as a function of solution type and surface functionality. In addition, studies were done to determine the structure of LRAP in solution and the structure of LRAP adsorbed onto surfaces using DLS and AFM.

2. Experimental methods

2.1. Protein synthesis and purification

Murine LRAP was synthesized by United Biochemical Research Inc., Seattle, WA as described previously (Shaw et al., 2004b). The protein was phosphorylated as shown in Table 1. Purity and identity were determined with HPLC and electrospray mass spectrometry.

2.2. Self-assembling monolayer (SAM) formation

N-hexadecanethiol (Aldrich, 92%) was purified by vacuum distillation. 16-Mercaptohexadecanoic acid was synthesized as described previously (Tarasevich et al., 2003). 11-Amino-1-undecanethiol, hydrochloride was obtained from Dojindo Laboratories. Polycrystalline gold for ellipsometry experiments was freshly deposited onto 15 mm diameter glass discs using a titanium or chromium adhesion layer. For AFM experiments requiring large atomically smooth single crystal terraces, mica substrates with deposited gold were obtained from Structure Probe Inc. (West Chester, PA). The gold was freshly deposited, hydrogen annealed, and sealed with argon in glass containers. The substrates were placed into 0.5–1 mM thiol solutions in absolute ethanol or hexane for at least 24 h. The amine thiol solutions were prepared in a nitrogen-purged glove box and contained 3 vol % triethylamine in nitrogen purged ethanol, by a method previously described to reduce the formation of multilayers (Wang et al., 2005). Samples were removed and cleaned in ethanol (CH_3), in nitrogen purged NH_4OH /ethanol solutions (NH_2), or acetic acid/ethanol solutions (COOH). Amine samples were used immediately upon removal from the glove box. The 16-mercaptohexadecanoic acid, *N*-hexadecanethiol, and 11-amino-1-undecanethiol SAMs were described by their end groups COOH , CH_3 , and NH_2 , respectively. By convention the end groups were given as the nonionized groups even though the groups may be protonated or deprotonated in solution. Advancing contact angles of water were typically 20° , 110° , and 30 – 40° for the COOH , CH_3 , and NH_2 SAMs, respectively.

2.3. Protein solutions and adsorption

LRAP was dissolved in 0.01 M HCl to fully solubilize the protein as evidenced by DLS studies. The protein was then diluted into buffer solutions to concentrations ranging from 0.2 mg/ml to 1 mg/ml. Two types of buffer solutions were used (1) “SCP”, 0.15 NaCl saturated with respect to hydroxyapatite and (2) phosphate buffered saline (PBS). The SCP buffer was

prepared by adding hydroxyapatite powder (Aldrich) to 0.15 M NaCl at pH 7.4, stirring for several days, and filtering out the particles. The calcium concentration was 7×10^{-5} M at saturation. These solutions have been developed previously for the adsorption studies of biomineralization proteins and peptides in the presence of calcium and phosphate at concentrations saturated with respect to hydroxyapatite and therefore undersaturated with respect to other phases such as octacalcium phosphate (OCP) and tricalcium phosphate (TCP) (Goobes et al., 2007; Shaw et al., 2008). The SCP buffer was studied by DLS and showed no evidence for the nucleation of calcium phosphate as would be expected for the non-super saturated solution. The PBS solutions were 0.14 M NaCl, 0.01 M KCl, 0.01 M Na_2HPO_4 , 0.002 M KH_2PO_4 and had a pH of 7.4. These solutions were used in comparison to the SCP solutions to study the role of calcium on adsorption. The SCP and PBS solution conditions were more similar to physiological conditions than other solutions such as Tris buffer. The protein solutions were adjusted to pH 7.4 using KOH. The concentrated protein solutions were then diluted down to various concentrations, as low as 0.001 mg/ml for the protein adsorption studies and as low as 0.10 mg/ml for the dynamic light scattering studies. SAM substrates were placed into the protein solutions (2–5 ml) for various time periods up to 24 h at ambient temperature. Most of the data presented here is for substrates exposed to protein solutions for 18–20 h. There were no changes in the pH of the solutions over the time course of the experiment. The substrates were removed and rinsed with a 12 ml stream of deionized water and dried in a stream of nitrogen. It was found that extended rinsing resulted in no significant changes in protein adsorption.

2.4. Single wavelength ellipsometry

Single wavelength ellipsometer (SWE) measurements were performed on a Rudolph Autoel II null ellipsometer using a wavelength of 632.8 nm and angle of incidence of 70°. The ellipsometric constants Δ and ψ were determined for the bare gold substrates soon after removal from the deposition chamber and new ellipsometric constants were determined after SAM formation. A 3-layer model (ambient/SAM/gold) was used to calculate the SAM thickness using a refractive index of $1.50 + 0i$ for the SAM (Parikh and Allara, 1992). Thicknesses of adsorbed protein layers were determined using a 4-layer model (ambient/protein/SAM/gold) assuming a refractive index of $1.50 + 0i$ for the protein, similar to methods described previously (Lestelius et al., 1997; Ortega-Vinuesa et al., 1998; Prime and Whitesides, 1993). The refractive index is an average of refractive indices typically found for proteins (1.4–1.6). (Prime and Whitesides, 1993). If the refractive index was 1.4, the adsorbed amounts would be 16% larger than the amounts determined using a refractive index of 1.5. The amounts were 11% smaller using a refractive index of 1.6.

The thicknesses were equivalent thicknesses, the true thickness of the protein times the surface coverage. The thicknesses were converted to concentrations, N ($\mu\text{g}/\text{cm}^2$), using a protein density of $1.37 \text{ g}/\text{cm}^3$ by the method of Stenberg and Nygren (1983). The adsorption amounts presented were the mean values $\pm$ standard deviation for at least three samples with five ellipsometry measurements per sample. SAM substrates exposed to buffer without protein were also prepared as controls.

2.5. Atomic force microscopy (AFM)

Atomic force microscopy was conducted in air and solution using a Digital Instruments Nanoscope IIIa Multimode system (Veeco Metrology, Santa Barbara, CA) equipped with a Quadrex module. The AFM head was placed in an enclosure lined with Sonex acoustic dampening foam (Illbruck, Minneapolis, MN) and placed on a vibration isolation table (Micro-g, TMC, Peabody, MA). Substrates with SAMs and substrates with LRAP adsorbed (after adsorption periods of 18–20 h) were imaged in tapping mode using TESP single beam silicon cantilevers having a nominal spring constant of $\sim 50 \text{ N/m}$ (Veeco Probes, Camarillo,

CA) Topography and phase images were simultaneously obtained at scan rates ranging from 1.5 Hz to 3.5 Hz and at amplitude setpoints ranging from 0.6 to 0.8 of the cantilever excitation free amplitude. Several experiments were also done in solution using a Nanoscope solution cell. Protein solutions were introduced into the solution cell for 1 h, flushed with buffer without protein, and images were obtained. There were no significant differences between dry state and wet state images. The dry state images tended to have higher resolution and were presented here.

Images from first scans were used as much as possible because the tip tended to become contaminated by the physisorbed protein over time. The images shown in this manuscript were unmodified except for tilt removal using a second-order plane fit in some cases. Graphic images were obtained using the Nanoscope software (Version 5.12r5) or by importing the raw data into ImageJ (rsb.info.nih.gov/ij/). The images were processed for brightness and contrast using Adobe Photoshop. The same height scale was used in comparing 1 μm scans of the bare substrates with substrates with adsorbates (0–20 nm). A height scale of 0–15 nm was used for the 300 nm scans. The heights and diameters of the adsorbed structures were measured using the section tool of the Nanoscope software. Diameters were measured as the full width half maximum (FWHM). The diameter measurements were uncorrected for tip broadening effects.

2.6. Dynamic light scattering

DLS measurements on the protein solutions were obtained using a Brookhaven Instruments 90 Plus equipped with a 657 nm 35mW laser. Time dependent fluctuations in the scattered intensity were measured using a BI-APD digital correlator. Protein solutions were analyzed in triplicate using a 90° scattering angle at 25.0 °C. The buffer solutions were filtered through 0.2 μm and 0.02 μm filters and were also analyzed by DLS. Standard NIST traceable polystyrene 22 nm $\pm$ 1.8 nm latex standards and a blank, 0.02 μm filtered DI ultrapure water (VWR), were also run as standards. Data was collected as coadded runs of 10 s to 2 min collected for a total of 10–20 min. The autocorrelation functions were deconvoluted to obtain size distributions using both the non-negatively constrained least squares fit (multiple pass NNLS) and the regularized LaPlace inversion (Contin) algorithms. The size distributions obtained from the NNLS algorithm were presented since the distributions were multimodal. The intensity of scattered light is proportional to the particle size to the sixth power which results in a higher scattered intensity for larger particles. The intensity weight distributions measured by DLS were converted to number weighted distributions using analysis software provided by Brookhaven.

3. Results

3.1. Solution characterization

3.1.1. DLS—The sizes of structures of LRAP in the SCP and PBS solutions at pH 7.4 at a range of solutions concentrations were studied. The data was obtained within 1 h after formation of the solutions and then at various time periods up to 24 h. A typical distribution of structures is shown in Fig. 1 for a 0.5 mg/ml solution of LRAP in the SCP buffer. The solution had LRAP structures with sizes of 171 $\pm$ 26 nm and a very small number of larger structures at ~1100 nm. The mean was determined by fitting the curves to a Gaussian distribution and the standard deviation was the width of the distribution. The sizes of the LRAP structures as a function of protein concentration are shown in Fig. 2. The small structures were around 150 nm with a slight trend toward increasing size with increasing LRAP solution concentration for the PBS solutions (Fig. 2a). For the SCP solutions (Fig. 2b), the structures also had a slight trend toward increasing size with increasing protein

concentration from 160 nm to 200 nm. There were no significant changes in the aggregates sizes over 18–20 h, the typical time period for adsorption studies.

3.2. Equilibrium adsorption amounts

3.1.2. Ellipsometry—The adsorption of LRAP was evidenced by changes in the ellipsometric constants, delta and psi, relative to the SAM surfaces. Controls of substrates exposed to buffers without protein showed no adsorbates. Adsorbed amounts were determined from the equivalent protein thicknesses obtained from the ellipsometric data after surfaces were exposed to protein solutions for 18–20 h. Adsorption kinetics experiments showed that a saturation limit of protein adsorption occurred by 30–60 min over the entire range of protein concentrations in solution. The adsorbed protein amounts increased with increasing solution concentration of the protein until a plateau region was reached in most cases as shown in Fig. 3. The slopes of the initial part of the adsorption curves from the PBS solutions were highest for the CH₃ surfaces > NH₂ >> COOH (Fig. 3b). LRAP adsorption from the SCP solutions (Fig. 3c and d) showed slopes of the adsorption curves going as CH₃ > NH₂ > COOH. Significantly higher amounts of adsorption occurred onto the COOH surfaces from SCP solutions compared to the PBS solutions. Although the slope of the COOH adsorption curve was lower than the slope of the NH₂ adsorption curve, adsorption onto the COOH surfaces continued to increase until the adsorption amounts became higher than the protein amounts on the CH₃ and NH₂ surfaces. The highest adsorption amounts for the CH₃ and NH₂ surfaces corresponded to ~1.6–1.8 nm in equivalent thickness. The highest measured adsorption amounts onto the COOH surfaces from the SCP solutions at 1000 µg/ml corresponded to ~2.0 nm in equivalent thickness.

The first-order Langmuir adsorption isotherm given by the equation:

$$N = N_{\max} \left(\frac{KC}{1 + KC} \right) \quad (1)$$

was fit to the adsorption data, where N is the protein concentration at the surface (mol/m²), C is the protein concentration in solution (mol/m³), K is the equilibrium binding constant (M⁻¹), and $N_{\max}$ is the maximum adsorption amount (mol/m²). The fits are shown in Fig. 3 and the values for the binding constants, K , and maximum adsorption amounts, $N_{\max}$ are shown in Table 2 for the CH₃, NH₂, and COOH surfaces in the PBS and SCP solutions. The binding constant, K , was significantly higher for the CH₃ surface compared to the other surfaces, in the order CH₃ >> NH₂ > COOH. The maximum adsorption amounts, $N_{\max}$, were similar for the CH₃ and NH₂ surfaces but were higher for the COOH surface from SCP solutions.

3.3. Adsorbate structure

3.1.3. AFM—AFM was used to examine the structures of the protein adsorbed onto the various self-assembled monolayers on gold on mica, a molecularly smooth surface. The surfaces were typically exposed to protein solutions for 18–20 h. Images of several surface and solution conditions studied are shown in Fig. 4. Fig. 4a showed a scan of COOH SAMs on gold on mica. Large 250–600 nm atomically smooth gold terraces (root mean square roughness values of ~0.2 nm) were separated by 2–10 nm step edges. LRAP adsorbates on COOH adsorbed from SCP solutions at 85 µg/ml concentration consisted of a relatively high coverage of very small adsorbates and several larger nanosphere-like structures overlying the smaller adsorbates (1 µm scan in Fig. 4b). The high resolution (300 nm) scan of LRAP adsorbed onto the COOH surfaces from SCP solutions (Fig. 4c) gave a better view of the small adsorbates. Fig. 4d showed a 1 µm scan and Fig. 4e showed a higher magnification

view of structures on NH₂ surfaces adsorbed from PBS solutions at 85 µg/ml concentration. These images also showed a relatively high coverage of small adsorbates and several larger nanospheres at low coverage. AFM studies of the other surface/solution conditions studied (LRAP adsorbed onto the CH₃ surfaces from PBS and SCP solutions and LRAP adsorbed onto NH₂ surfaces from SCP solutions) showed similar structures as the adsorbates shown in Fig. 4 – a predominance of very small structures with no significant adsorption of larger nanospheres.

The sizes of the small adsorbates were determined by measuring the diameters at full width half maximum (FWHM) and the heights from cross sections of the images. It is well known, however, that AFM measurements of structures that are smaller than the radius of curvature of the tip (10–20 nm) are overestimated by several times due to the tip broadening effect (Garcia and Perez, 2002; Grabar et al., 1997; Yang et al., 2001). The large tip exaggerates the lateral dimensions as it traces over a structure smaller than the radius of the tip. Also, the height of soft structures such as proteins can be underestimated up to six times because of the nonlinear dynamic response of the oscillating cantilever (Round and Miles, 2004; San Paulo and Garcia, 2000, 2002). Under the best conditions, the measured heights of biomolecules such as DNA have been found to be 0.6 of their true dimension. In spite of the inaccuracies, AFM sizes were determined for comparison purposes and to be calibrated with more accurate measurements such as ellipsometry. It was found that the small adsorbates had similar sizes on the various surfaces and the two solution conditions and were in the size range of ~10–15 nm × 0.5–0.75 nm (diameter × height) and the larger structures were ~20–40 × 7–10 nm. The small adsorbates had very shallow height relief because of the height underestimation and were difficult to image and resolve. Although the diameters were large because of the tip broadening effect, these structures were clearly much smaller than the larger 30 × 7 nm structures. We believe that the larger structures were nanospheres. Based on the equivalent thicknesses determined by ellipsometry (in the range of 1.5–2.0 nm), we suggest that the smaller adsorbates were subnanosphere-sized structures such as monomers or dimers. If the radius of curvature of the tip was 10–20 (typical range according to the manufacturer), feature diameter was 2–4 nm, and feature height was 2 nm, a simple geometric model would predict that the measured feature size due to tip broadening would be 14–21 nm (7–11 nm if the diameter is measured at the full width half maximum). The predicted AFM sizes for 2–4 nm diameter structures, therefore, are consistent with the measured sizes.

Adsorbates from solutions at higher LRAP concentrations (1 mg/ml) were also studied and showed similar structures as the adsorbates at lower protein concentration – small subnanosphere-sized adsorbates at high coverage (Figs. 4f and S1). However, in contrast to the lower concentration protein solutions, there was a higher concentration of nanospheres overlying the smaller adsorbates. The nanospheres were 20–40 nm in diameter and in some cases they were clustered together to form 100 nm to 200 nm diameter aggregates, many of which were chain-like in appearance.

4. Discussion

4.1. Adsorption mechanism

We studied the adsorption of LRAP onto self-assembled monolayers with different functional groups in order to develop a better understanding of the fundamental interactions involved in protein adsorption. The ellipsometry and AFM studies revealed that LRAP adsorbed onto self-assembled monolayers as subnanosphere-sized structures, structures that may be in the size range of individual LRAP monomers or dimers. Although monomers and/or dimers were observed on the surfaces, there was no evidence for the presence of these

structures in solution by DLS in both the PBS and SCP solutions at a range of protein concentrations studied.

One possible explanation for the presence of these structures on the surface is that monomers and dimers may be present in solution even though they are not detected by DLS and adsorb preferentially over the larger nanosphere aggregates. This mechanism is shown in the schematic in Fig. 5a. The intensity of scattered light is proportional to the particle diameter to the sixth power, favoring the detection of larger structures. However, we observed no evidence for monomers or dimers in solutions that were 5 μm and 0.4 μm filtered to remove the larger aggregates. Solutions that were 0.2 μm filtered showed no scattered intensity, indicating that most of the LRAP structures were larger aggregates removed by filtering. At the present time, we do not have any evidence for the presence of monomer or dimer structures in the pH 7.4 solutions. This is consistent with a large number of studies of quaternary structures of the full-length amelogenin protein at pH values in the range of 6–8 using DLS (Aichmayer et al., 2005; Du et al., 2005; Moradian-Oldak et al., 1998b, 1994; Petta et al., 2006), small angle X-ray scattering (SAXS) (Aichmayer et al., 2005), and small angle neutron scattering (SANS) (Aichmayer et al., 2005). Small structures in the size range of monomers to dimers have been observed in solution, but primarily in acidic solutions less than pH 4 (Aichmayer et al., 2005; Matsushima et al., 1998; Petta et al., 2006) and from less polar solvents such as 60% acetonitrile in water (Du et al., 2005). We did a few adsorption studies from solutions of LRAP dissolved in 60% acetonitrile or acetic acid at pH 3 and observed monomer/dimer-sized structures, similar to the small structures found in this study at pH 7.4.

Recent DLS studies were done on dilute solutions of full-length amelogenin (5 $\mu\text{g}/\text{ml}$) in calcium or phosphate containing solutions at pH 5.6–6.8 at 37 °C (Wang et al., 2007). Relatively small oligomers of amelogenin (as small as 6.6 nm diameter) were occasionally observed when DLS data was obtained at short 10 s acquisition times. When we obtained DLS data from our LRAP solutions at similar short acquisition times, however, we were unable to detect any structures smaller than the nanosphere aggregates.

Another possible mechanism for LRAP adsorption is that small monomers or dimers “shed” or disassemble from the larger nanosphere aggregates present in solution as shown schematically in Fig. 5b. Monomers may “peel” away from the nanospheres onto the surfaces reversing the process of LRAP self-assembly in solution. This type of behavior would be similar to the way phospholipid vesicles interact at surfaces. Phospholipids form spherical vesicles in solution but they disassemble or “spread” onto surfaces to form planar lipid monolayers and bilayers (Ohki et al., 1988; Plant, 1993). Vesicles disassemble onto hydrophobic surfaces by interactions of the nonpolar alkyl chains of the lipid with the surface (Meuse et al., 1998). Electrostatic interactions between the polar head group of the lipid and hydrophilic surfaces such as silica and mica surfaces (Richter et al., 2003; Stelzle et al., 1993) can also cause phospholipids vesicles to disassemble. We propose this as a possible mechanism because of the lack of evidence for monomer/dimer structures in our solutions. Further studies on the quaternary structure of LRAP in solution and the adsorbate structure on the surface will be necessary, however, in order to conclusively determine the adsorption mechanism. We have previously found that amelogenin (both rp(H)M180 and rM179) adsorbed onto fluoroapatite and COOH SAM surfaces as monomers to small oligomers (Tarasevich et al., 2009a,b) even though only larger nanospheres were detected in the solutions by DLS. Nanospheres and aggregates of nanospheres initially adsorbed onto the surfaces and then were displaced by the smaller structures over time. This suggests that both LRAP and full-length amelogenin can interact at surfaces as small subnanosphere structures.

4.2. Solution structure

DLS studies were done to determine the LRAP structure in SCP and PBS solutions. To our knowledge, there have been no previous studies of LRAP particle sizes in solution. The DLS data showed that both the SCP and PBS solutions contained structures in the size range of 150–200 nm in diameter. AFM studies showed the adsorption of 20–40 nm diameter nanospheres and aggregates of nanospheres, especially at high LRAP concentrations (1 mg/ml). This indicates that the large structures observed by DLS were clusters or aggregates of nanospheres. The aggregation of nanospheres is well known and has been observed by a number of previous studies of full-length amelogenin using DLS, small angle neutron scattering (SANS), and small angle X-ray scattering (SAXS) (Aichmayer et al., 2005; Moradian-Oldak et al., 1998b, 1994; Petta et al., 2006). We believe that nanosphere clustering may have been promoted in our studies since the protein was dissolved in acid and was brought through the isoelectric point of amelogenins at around pH 6 before reaching pH 7.4. This resulted in cloudiness in the solutions which has been associated with nanosphere aggregation.

4.3. Adsorbate quaternary structure

The AFM studies showed the presence of small adsorbate structures on the self-assembled surfaces, structures that were much smaller than the LRAP nanospheres present in solution. The atomic smoothness of gold on mica greatly aided in the detection of these structures as it was difficult to observe the relatively small AFM height relief on rough surfaces such as SAMs on polycrystalline gold (see Fig. S1). These adsorbates appeared to be present at relatively high coverage.

Although we know that the small adsorbates are not nanospheres, it is not clear whether the adsorbates are monomers or dimers or other small oligomers because the size of the LRAP monomer has been previously undetermined. Earlier we performed neutron reflectivity studies of LRAP adsorbed onto surfaces to obtain scattering length density profiles as a function of distance away from the surface (Shaw et al., 2004b). These studies showed that the adsorbed LRAP protein had a true thickness of 2–2.5 nm. In addition, residues near the C-terminus of bovine LRAP (L₄₂ to A₄₈) were labeled with deuterium and were found to be oriented toward the surface as evidenced by a pronounced increase in the scattered length density in the near surface region. Only one distinct deuterated region was observed in the profile indicating that LRAP was absorbing as a structure that had the thickness of a monomer but not a dimer. This suggests that LRAP has a monomer size of ~2–2.5 nm. We believe that this is a reasonable estimate for an LRAP monomer because it is smaller than the size of the full-length amelogenin monomer, estimated to be ~4.6 nm in diameter (Du et al., 2005). A smaller size would be expected for the lower molecular weight (6.8 kDa) LRAP compared to the full-length amelogenin (~20 kDa). The adsorbates on the CH₃ and NH₂ SAM surfaces had equivalent ellipsometric thicknesses of ~1.5–1.8 nm which would be consistent with the adsorption of a structure that is the thickness of a 2.5 nm diameter monomer at 0.6–0.72 coverage. The neutron reflectivity and ellipsometry data suggest that the adsorbed structures are of monomer thickness, however, it is possible that the adsorbed structures are dimers or other small oligomers that lie with their long axis parallel to the substrate. This possibility is shown in the schematic in Fig. 5. A previous study of the adsorption of LRAP showed no nanospheres adsorbed onto fluoroapatite and silica surfaces from 0.1 mg/ml solutions but significant concentrations of 10–30 nm diameter nanospheres adsorbed from 1 mg/ml and 7.5 mg/ml solutions (Habelitz et al., 2006). This result would be consistent with our study which found higher concentrations of adsorbed nanospheres at 1 mg/ml concentration compared to the lower concentrations. Our DLS studies indicate that nanospheres were present in solutions at low LRAP concentrations even though they did not adsorb significantly. Higher concentrations of nanospheres in the 1 mg/ml solutions were

necessary to promote the adsorption of nanospheres and nanosphere aggregates as multilayers over the underlying monomer/dimer adsorbates.

4.4. Effect of surface functionality

The ellipsometry studies showed that some degree of LRAP adsorption occurred onto the NH_2 , COOH , and CH_3 surfaces. Based on the equilibrium binding constants, K , determined from the adsorption data, LRAP had the highest affinity for the CH_3 surfaces followed by the NH_2 and then the COOH surfaces. LRAP contains a large central hydrophobic region and a charged domain at the C-terminus. It is expected that the large central hydrophobic region would promote the adsorption of LRAP onto nonpolar CH_3 surfaces due to the hydrophobic interaction. Most proteins have high affinities for nonpolar surfaces because of the hydrophobic effect. For example, IgG and albumin have been found to adsorb readily to polyethylene surfaces with binding constants of $\sim 400 \times 10^5 \text{ M}^{-1}$ (Young et al., 1988a). These binding constants are higher than the binding constants for LRAP adsorbed onto CH_3 SAMs shown in Table 2 ($34.3 \times 10^5 \text{ M}^{-1}$ and $41.9 \times 10^5 \text{ M}^{-1}$). The higher constants may be attributed to the higher molecular weight of IgG (150 kDa) and albumin (66 kDa) compared to LRAP (6.8 kDa) resulting in a higher number of potential binding sites per mole of protein. The adsorption affinities of proteins of different molecular weights onto hydrophobic surfaces have been previously compared by expressing the equilibrium constants in terms of mass units (Young et al., 1988a,b). This allows the normalization of the constants to units that correspond more closely to the number of potential hydrophobic binding sites and not the number of bound molecules. When compared in this way, the adsorption constants of IgG and albumin onto polyethylene were $390 \text{ cm}^3/\text{mg}$ and $1300 \text{ cm}^3/\text{mg}$, respectively, compared to the adsorption constant of $571 \text{ cm}^3/\text{mg}$ for LRAP on the CH_3 SAMs. This comparison suggests similar adsorption affinities per potential binding sites.

The protein also had a relatively high adsorption affinity for NH_2 surfaces which may be promoted by interactions with the negatively charged residues in the C-terminal domain such as glutamic acid (E) and aspartic acid (D) (shown in Table 1). It would be expected that the amine surfaces would have some degree of protonation and an overall positive charge at pH 7.4. This suggests that adsorption onto the amine surfaces is promoted by primarily electrostatic interactions. Recent solid-state NMR studies have shown that the C-terminal domain lies down flat at the surface indicating the potential for adsorption interactions with multiple charged sites (Shaw et al., 2008). Previous research has found that amelogenin adsorption was promoted onto polyelectrolytes containing amine groups, also suggesting the importance of electrostatic interactions between the C-terminal domain and positive surfaces (Gergely et al., 2007).

There was significantly less adsorption onto the COOH surfaces from the PBS solutions. This is not surprising considering that the entire LRAP molecule and the hydrophilic C-terminal domain are expected to have net negative charges at pH 7.4 and the COOH SAM is also negatively charged at that pH. The COOH SAM has a pK of pH 6–7 (Bain and Whitesides, 1989) so the surface will consist of a mixture of COOH and COO^- sites. Significant increases in protein adsorption occurred when LRAP was in the calcium containing SCP solutions. Adsorption may occur by calcium bridging between surface COOH sites and COOH sites of the aspartic acid and glutamic acid protein residues. Protein adsorption by calcium bridging is well known and is promoted by divalent calcium interacting with two monovalent COO^- sites, one on the surface and one on the protein (Klinger et al., 1997; Miklavcic et al., 1996; van Oss, 2006; Wassell and Embery, 1996).

A previous study found that the binding constant determined from the Langmuir model for full-length amelogenin, M179, adsorbed onto hydroxyapatite surfaces was $19.7 \times 10^5 \text{ M}^{-1}$

(Bouropoulos and Moradian-Oldak, 2003). In this case, since the C-terminal domain is expected to be involved in binding interactions with the surface and there is one C-terminal domain per molecule for both amelogenin and LRAP, the binding constant in molar units is a more appropriate value for comparison purposes. The mole normalized constant was higher than the constants we obtained for LRAP adsorbed onto the hydrophilic NH_2 ($4.5 \times 10^5 \text{ M}^{-1}$ from PBS and $2.8 \times 10^5 \text{ M}^{-1}$ from SCP) and COOH ($0.5 \times 10^5 \text{ M}^{-1}$ from SCP) self-assembled surfaces. The charged regions of LRAP and amelogenin that may be involved in electrostatic binding interactions with surfaces are highly conserved between the two proteins. The differences in binding affinities, therefore, may reflect differences in surface chemistry between the hydroxyapatite surface and the NH_2 and COOH surfaces. Hydroxyapatite has a mixture of positive calcium and negative phosphate sites (Wallwork et al., 2001) compared to the singly charged NH_2 and COOH surfaces. This suggests that a mixture of charges may promote higher adsorption affinities compared to singly charged surfaces, perhaps by promoting multiple binding interactions with the C-terminus which contains a mixture of positive (2 lysine, 1 arginine) and negative (four glutamic acid, two aspartic acid) sites.

4.5. Relevance to previous studies

We studied the adsorption of LRAP onto self-assembled monolayers because these surfaces make very good model systems to study the fundamentals of how surface chemistry can affect adsorption interactions. These surfaces have advantages in being smooth enough for neutron reflectivity studies and can be formed on mica resulting in atomically smooth surfaces for AFM studies. Although these surfaces have provided very useful information and allowed the detection of the very small LRAP monomeric adsorbates by AFM, adsorption onto calcium phosphate is of greater interest in understanding how LRAP interacts with surfaces as a model for amelogenin. We would expect that the adsorption behavior onto the charged, hydrophilic HAP surface may be similar to the adsorption behavior onto the hydrophilic surfaces, COOH and NH_2 . Initial studies of the adsorption of LRAP onto single crystal FAP surfaces have shown similar monomer/dimer adsorbates as we found on the SAMs.

LRAP adsorbed as monomers/dimers onto the self-assembled surfaces with no significant adsorption of nanosphere overlayers until protein concentrations of 1 mg/ml were achieved. The adsorption of monomers is suggested by our previous studies of the tertiary structure of LRAP using neutron reflectivity as discussed above (Shaw et al., 2004b). The adsorption of monomers is also consistent with previous studies of the secondary structure of LRAP adsorbed onto hydroxyapatite using solid-state NMR (Shaw and Ferris, 2008; Shaw et al., 2004a, 2008). Solid-state NMR studies determined the intersite spacings between ^{13}C and ^{15}N isotopically labeled LRAP residues in the C-terminal domain and naturally occurring ^{31}P in the hydroxyapatite surface. For example, it was found that the residues of A₄₆, A₄₉, and A₅₂ were positioned from 5.8 Å to 7 Å away from the hydroxyapatite surface (Shaw et al., 2008). These spacings are consistent with the adsorption of an LRAP monomer, since the adsorption of a dimer or nanosphere would result in larger intersite spacings.

Like LRAP, we previously observed that full-length amelogenin adsorbed onto fluoroapatite, CH_3 , and COOH SAMs as subnanosphere-sized structures that were monomers to oligomers in size (Tarasevich et al., 2009a,b). This indicates that both LRAP and full-length amelogenin behave similarly at interfaces. Although the adsorption mechanism is open to interpretation and continues to be investigated, our work suggests that subnanosphere-sized quaternary structures of amelogenin and LRAP are important at interfaces in *in vitro* models. Although the biological role of LRAP is currently under debate, our studies showing adsorption onto hydrophilic surfaces suggest that LRAP could

function by adsorbing onto enamel crystals, modulating the size and habit of crystallites and promoting their unusually high aspect ratio. The monomers and dimers may affect crystallite size and shape while the larger nanosphere structures may have a role in controlling spacing between the crystallites and promoting the highly interwoven structures.

It remains to be seen if the LRAP monomers or dimers are present in vivo within growing enamel and if they have functions that are different than amelogenin nanospheres that have been observed in vivo. Our in vitro models, however, have provided important insights into the adsorption behavior of LRAP and will lead to a further understanding of the size of the LRAP monomer, the size and structure of the small LRAP adsorbates, the adsorbate structure onto hydroxyapatite surfaces, and differences in behavior and function of monomers compared to nanospheres.

5. Conclusions

Studies of the adsorption of LRAP onto self-assembled surfaces revealed that monomers or dimers adsorbed onto the surfaces even though only aggregates of nanospheres were detected in solution by DLS. There was no significant adsorption of nanospheres at lower concentrations but nanospheres adsorbed over the underlying monomers at solution concentrations of 1 mg/ml protein. Determinations of the equilibrium adsorption constants from adsorption isotherms determined by ellipsometry showed that LRAP had the highest affinity for CH₃ SAMs followed by NH₂ SAMs. This indicates the importance of both hydrophobic interactions and electrostatic interactions depending on the surface studied. There was no significant adsorption of LRAP onto COOH surfaces unless calcium was present in solution suggesting a calcium bridging mechanism. Although the adsorption mechanism is currently under investigation, this work reveals the importance of subnanosphere-sized structures of amelogenins at interfaces and suggests that the monomer/dimer as well as the nanosphere quaternary structure may have importance biologically.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH-NIDCR Grant DE-015347. This research was performed at Pacific Northwest National Laboratory, operated by Battelle for the US-DOE. A portion of the research was performed in the EMSL, a national scientific user facility sponsored by the DOE-OBER at PNNL.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:
10.1016/j.jsb.2009.10.007.

Fig. 1. Dynamic light scattering studies of 0.5 mg/ml LRAP in SCP solutions at pH 7.4 showing large structures averaging 170 nm diameter.

Fig. 2. DLS determined sizes of LRAP structures at various concentrations in (a) PBS solutions at pH 7.4 and (b) SCP solutions at pH 7.4. Error bars represent the standard error of the Gaussian distribution.

Fig. 3.

Adsorption amounts versus solution protein concentration for LRAP adsorbed onto COOH, NH₂, and CH₃ surfaces after 18 h from (a) PBS solutions up to 1 mg/ml, (b) expanded view of PBS data up to 0.1 mg/ml, (c) SCP solutions up to 1 mg/ml, and (d) expanded view of SCP data up to 0.1 mg/ml.

Fig. 4.

Tapping mode AFM images (a) 1 μm scan of COOH SAMs on gold on mica, (b) 1 μm scan of adsorbates on COOH SAM from SCP solutions at 85 $\mu\text{g/ml}$ after 18 h showing a high coverage of monomer/dimer adsorbates (small arrow) with several larger nanospheres (large arrow), (c) high resolution 300 nm scan showing monomer/dimer adsorbates (arrow) on COOH SAM from SCP solutions at 85 $\mu\text{g/ml}$, (d) 1 μm scan of adsorbates on NH_2 surface from PBS solutions at 85 $\mu\text{g/ml}$ after 18 h showing a relatively high coverage of small adsorbates (small arrow) with several larger nanospheres (large arrow), (e) higher magnification picture of the image in 4d giving a better view of the small adsorbates (small arrow), and (f) 1 μm scan of adsorbates onto a NH_2 surface from 1 mg/ml LRAP in SCP solution showing nanospheres and aggregates of nanospheres (large arrows) overlying the smaller monomer/dimer adsorbates (small arrow). The ellipsometry data suggests that the small adsorbates are of monomeric thickness, ~ 2 nm. The small adsorbates have a small height relief (0.5–0.75 nm) because the AFM heights are underestimated (see text) but the height relief can be clearly seen in relationship to the bare SAM surfaces. The monomer/dimer adsorbates are 10–15 nm $\times$ 0.5–0.75 nm (diameter $\times$ height) in contrast to the nanospheres which are 20–40 nm $\times$ 7–10 nm. It is easiest to differentiate the monomer/dimers from the nanospheres by the height disparity.

Fig. 5. Schematic of possible mechanisms for the adsorption of monomeric structures onto the SAM surfaces involving (a) the presence of monomers or dimers in solution and preferentially adsorbing onto the surfaces as monomers or dimers oriented parallel to the surface and (b) the “shedding” or disassembly of monomers or dimers from nanospheres and aggregates of nanospheres onto the surface.

Table 1

Amino acid sequences for murine LRAP and amelogenin showing the conservation of primary structure in the N- and C-terminal region. Charged amino acids are in bold typeface. The central part of amelogenin is indicated by #.

LRAP	MLPPHCGSPGYINL _p SYEVL _T TPL K WYQSMIRQPP _L SPIL _{PEL} PLEAWPAT DKTKREEVD
Amelogenin	MLPPHCGSPGYINL _p SYEVL _T TPL K WYQSMIRQ _# PLSPIL _{PEL} PLEAWPAT DKTKREEVD #YPSYGYEPMGGWLHHQIIPVLSQQHPFSHTLQPHHHLPVVPAQQPVAPQQPMMPVPGHHSMITPTQHHQPNIPPSAQQPFQQPFQQAIPQSQSHQPMQPSPLHPMQPLA PQPPLPLFSMQ

Table 2

Experimentally determined thermodynamic parameters for the adsorption of LRAP onto surfaces from the Langmuir model.

Solution	Surface	K ($10^5/\text{M}$)	N_{max} (10^{-7} mol/m ²)
PBS	CH ₃	34.3	2.8
	NH ₂	4.5	3.6
	COOH	–	–
SCP	CH ₃	41.9	3.0
	NH ₂	2.8	4.2
	COOH	0.5	6.4