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# The leucine-rich amelogenin protein (LRAP) is primarily monomeric and unstructured in physiological solution

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ABSTRACT

Amelogenin proteins are critical to the formation of enamel in teeth and may have roles in controlling growth and regulating microstructures of the intricately woven hydroxyapatite (HAP). Leucine-rich amelogenin protein (LRAP) is a 59-residue splice variant of amelogenin and contains the N- and C-terminal charged regions of the full-length protein thought to control crystal growth. Although the quaternary structure of full-length amelogenin in solution has been well studied and can consist of self-assemblies of monomers called nanospheres, there is limited information on the quaternary structure of LRAP. Here, sedimentation velocity analytical ultracentrifugation (SV) and small angle neutron scattering (SANS) were used to study the tertiary and quaternary structure of LRAP at various pH values, ionic strengths, and concentrations. We found that the monomer is the dominant species of phosphorylated LRAP (LRAP(+P)) over a range of solution conditions (pH 2.7–4.1, pH 4.5–8, 50 mmol/L(mM) to 200 mM NaCl, 0.065–2 mg/mL). The monomer is also the dominant species for unphosphorylated LRAP (LRAP(−P)) at pH 7.4 and for LRAP(+P) in the presence of 2.5 mM calcium at pH 7.4. LRAP aggregates in a narrow pH range near the isoelectric point of pH 4.1. SV and SANS show that the LRAP monomer has a radius of ~2.0 nm and an asymmetric structure, and solution NMR studies indicate that the monomer is largely unstructured. This work provides new insights into the secondary, tertiary, and quaternary structure of LRAP in solution and provides evidence that the monomeric species may be an important functional form of some amelogenins.



## 1. Introduction

The amelogenin proteins are necessary for the formation of tooth enamel, representing 90% of the proteins present in enamel fluid (Termine et al., 1980; Gibson et al., 2001). Humans with mutations in the *AMELX* gene and knock-out mice engineered to have *AMELX* null mutations have highly defective and disorganized enamel structure (Gibson, 2011). Amelogenin proteins are thought to function as a matrix to guide the mineralization of HAP extracellularly because amelogenin nanospheres have been observed along HAP crystallites in immature enamel (Fincham et al., 1995). *In vitro* studies have suggested that amelogenin has roles in initiating nucleation (Tarasevich et al., 2007), controlling growth (Iijima and Moradian-Oldak, 2004), and affecting the spacing of crystallites (Moradian-Oldak et al., 1998a).

Leucine-rich amelogenin protein (LRAP) is a 59-residue splice variant of amelogenin (Fig. 1) (Gibson et al., 1991). Because LRAP appears within the enamel fluid with amelogenin, it has been thought that the protein may have a role in enamel formation (Fincham et al., 1999). *In vivo* studies have shown that LRAP is localized within the extracellular matrix of growing enamel (Gibson et al., 1995) and *in vitro* studies have shown that LRAP can control HAP crystal formation (Le Norcy et al., 2011) suggesting that LRAP, like amelogenin, may have an extracellular matrix function in controlling enamel crystal growth. More recent studies have provided evidence that LRAP may promote enamel growth by acting as a cell signaling molecule, affecting ameloblast differentiation and protein expression. For example, LRAP partially rescued the null amelogenin mouse phenotype (Gibson et al., 2009, 2011), increased enamel growth in tooth explants (Ravindranath et al., 2007) and promoted the differentiation of human enamel organ epithelial cells (Le et al., 2007). LRAP that was overexpressed

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in transgenic murine models affected ameloblast differentiation and upregulated amelogenin, MMP-20, and SATB1 proteins (Stahl et al., 2013).

Although LRAP has a role in enamel formation, recent studies have shown that LRAP can also function as a cell signaling protein to promote differentiation of mesenchymal cells (Veis et al., 2000; Warotayanont et al., 2008). LRAP has been found to promote osteogenesis of rat muscle fibroblasts (Veis et al., 2000), cementoblasts (Boabaid et al., 2004), and mesenchymal stem cells (Warotayanont et al., 2009; Wen et al., 2011). A porcine enamel matrix derivative has been shown to have therapeutic applications in promoting the regeneration of cementum and bone in periodontal tissue (Hammarstrom, 1997; Heijl et al., 1997; Sculean et al., 2008) and LRAP has been shown to be the main factor within enamel matrix derivatives to promote osteogenesis (Amin et al., 2012).

In spite of the importance of LRAP as an enamel former, osteogenic protein, or regenerative agent, very little is known about its tertiary and quaternary structure. It will be difficult to fully understand LRAP's biological function or therapeutic potential without a better grasp of its structure. The dominant quaternary structure of full-length amelogenin in solution is the "nanosphere," aggregates of amelogenin monomers that are 20–60 nm in diameter (Moradian-Oldak, 2001). The nanospheres are considered to be self-assemblies because they are believed to have a hierarchical structure consisting of highly ordered oligomers (Fang et al., 2011). Nanospheres are present in solutions with pH values greater than 6.0 and salt concentrations ranging from 50 mmol/L (mM) to 200 mM (Moradian-Oldak et al., 1994, 1998). Oligomers have also been detected in solutions at pH 5.5 ranging in size from dimers to octamers depending on the protein concentration (Bromley et al., 2011).

Recently we found that phosphorylated LRAP exists as a monomer in 150 mM NaCl, pH 7.4 solutions (Tarasevich et al., 2013). Since this quaternary form is in contrast to the nanospheres observed for full-length amelogenin at pH 7.4, we wanted to further investigate the quaternary structure of LRAP over a range of solution conditions including those found in developing enamel (Sasaki et al., 1991; Robinson et al., 1995). LRAP was studied in solutions ranging from pH 3.0 to 8.0, 50 to 200 mM NaCl, and concentrations from 0.065 to 2 mg/mL. SV was used to determine the distribution of protein species present in the solutions (Lebowitz et al., 2002). SV has several advantages over other analysis techniques, such as dynamic light scattering (DLS), because it uses a centrifugal field to separate species from monomers to large aggregates based on the species' masses and shapes, whereas DLS relies on a mathematical separation. Although DLS is well suited for the measurement of sizes in monodisperse systems, it is not as accurate in characterizing polydisperse systems. Light scattering intensity has a size dependence of radius to the sixth power ($r^6$) so that large structures can dominate scattering and prevent the detection of smaller structures (Filipe et al., 2010). Also, DLS was not able to resolve particle populations that differed in size by a factor of 2 or less (Filipe et al., 2010). In contrast, SV can directly quantify the proportion of different species in solution on a weight basis, since SV typically uses UV absorbance to monitor sample separation. SV and SANS were also used to determine the size and shape of the LRAP monomers and solution NMR was used to study LRAP secondary structure.

MPLPPHPGSP GYINLSYEVL TPLKWYQSMI RQPYPSYGYE PMGGWLHHQI
IPVLSQQHPP SHTLQPHHHL PVVPAQQPVA PQQPMMPVPG HHSMTPTQHH
QPNIPPSAQQ PFQQPFQPQA IPPQSHQPMQ PQSPLHPMQP LAPQPPLPPL
FSMQPLSPIL PELPLEAWPA TDKTKREEVD

**Fig.1.** Primary amino acid sequence of murine amelogenin with the basic and acidic residues colored blue and red, respectively. The splice variant LRAP is composed of the N-terminal 33 and C-terminal 26 residues, shaded yellow. Both full-length amelogenin and LRAP are post-translationally modified by side chain phosphorylation of S16, colored green. The serine at position 16 was either phosphorylated (LRAP(+P)) or non-phosphorylated (LRAP(−P)).

## 2. Materials and methods[1]

### 2.1. Materials

Deuterium oxide (99.9%) was purchased from Cambridge Isotopes Laboratories and used as received. All other chemicals were reagent grade and obtained from Sigma–Aldrich.

### 2.2. Solid-phase LRAP(+P) and LRAP(−P) synthesis

Murine LRAP with phosphoserine (LRAP(+P)) and normal serine (LRAP(−P)) at position 16 (Fig. 1) was synthesized using solid-phase methods by the Protein Chemistry Technology Center, University of Texas (Dallas, TX) for SV, SANS, and zeta potential experiments. Each sample was purified by reverse phase HPLC using buffer A, 0.1% trifluoroacetic acid in water and buffer B, 0.1% trifluoroacetic acid in acetonitrile. LRAP eluted at 54% B. Mass spectroscopy was used to characterize the purity and molecular weight of the proteins. After purification, proteins were lyophilized for storage until ready for use.

### 2.3. Recombinant LRAP(−P) (rLRAP(−P)) synthesis

To obtain uniformly $^{13}C$- and $^{15}N$-labeled LRAP samples for solution NMR studies it was necessary to use recombinant methods. The protein, rLRAP(−P) containing a 12-residue (MRGSHHHHHHGS-) N-terminal tag, was prepared for the studies conducted in acetic acid at pH 2.8 as described previously (Buchko et al., 2010). For the studies conducted in SCP solution at pH 7.4, rLRAP(−P) was prepared with only a 4-residue (GPGS-) N-terminal tag as described in the Supplementary section.

### 2.4. LRAP solution formation

A stock solution of LRAP was dissolved in Millipore purified water at 5–10 mg/ml and stored at 4 °C overnight. The solution was centrifuged at 11,000 rpm for 30 min and the upper fraction decanted. The concentration of the stock solution was determined by measurement of the UV absorbance at 275 nm using an extinction coefficient of 15,470 ($M^{-1}$ $cm^{-1}$). Saturated calcium phosphate (SCP) solutions were prepared containing various NaCl concentrations (50–300 mM) and pH values (5.0–8.0). They were prepared by adding HAP powder to the NaCl solution, adjusting the pH, stirring for several days, and filtering out any particles as described previously (Shaw et al., 2004). A SCP solution is being used because it has a small amount of dissolved calcium and phosphate in the solution making it more relevant to the *in vivo* enamel forming environment. The amounts of calcium and phosphate do not exceed the saturation limit so there is no calcium phosphate precipitation. The LRAP stock solution was diluted into the various SCP solutions at the appropriate pH to obtain solutions containing 50, 150, and 200 mM NaCl. The solution pH values were initially ~pH 3.0 (due to formic acid in the lyophilizing solution) and were readjusted to values ranging from pH 3.0 to pH 8.0 using dilute

[1] Certain commercial equipment, instruments, or materials are identified in this paper to foster understanding. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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