Effect of phosphorylation on the interaction of calcium with leucine-rich amelogenin peptide

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Amelogenin undergoes self-assembly and plays an essential role in guiding enamel mineral formation. The leucine-rich amelogenin peptide (LRAP) is an alternative splice product of the amelogenin gene and is composed of the N terminus (containing the only phosphate group) and the C terminus of full-length amelogenin. This study was conducted to investigate further the role of phosphorylation in LRAP self-assembly in the presence and absence of calcium using small angle X-ray scattering (SAXS). Consistent with our previous dynamic light-scattering findings for phosphorylated (+P) and non-phosphorylated (−P) LRAP, SAXS analyses revealed radii of gyration (Rg) for LRAP(−P) (46.3–48.0 Å) that were larger than those for LRAP(+P) (25.0–27.4 Å) at pH 7.4. However, added calcium (up to 2.5 mM) induced significant increases in the Rg of LRAP(+P) (up to 46.4 Å), while it had relatively little effect on LRAP(−P) particle size. Furthermore, SAXS analyses suggested compact folded structures for LRAP(−P) in the presence and absence of calcium, whereas the conformation of LRAP(+P) changed from an unfolded structure to a more compact structure upon the addition of calcium. We conclude that the single phosphate group in LRAP(+P) induces functionally important conformational changes, suggesting that phosphorylation may also influence amelogenin conformation and protein–mineral interactions during the early stages of amelogenesis.

Enamel is the most highly mineralized vertebrate tissue, composed of ~96% mineral and 4% organic material and water. During amelogenesis, the ameloblast secretes matrix proteins and is responsible for creating and maintaining an extracellular environment favorable for mineral deposition (1). Amelogenin, the predominant enamel matrix protein, has been shown to undergo self-assembly to form spherical or oblate-shaped nanoparticles (2–4), as well as elongated structures (5–7), and is believed to play an essential role in guiding the formation of ordered arrays of apatitic crystals during enamel development (5, 8–12). In particular, both the N-terminal domain (containing the only phosphate group on serine 16) and the hydrophilic C-terminal domain of full-length amelogenin (Fig. 1) have been shown to be critical for proper enamel formation (13–16). It is also believed that during enamel mineral growth, the concentration of free calcium ions is regulated, in part, by the binding of calcium to enamel proteins and their proteolytic cleavage products (17, 18).

The leucine-rich amelogenin peptide (LRAP), a 56-amino-acid alternative splice product of the amelogenin gene found throughout amelogenesis and composed of the first 33 N-terminal and the last 23 C-terminal amino acids of full-length amelogenin (Fig. 1), has recently been shown by us to share similar behavioral properties with amelogenin with respect to self-assembly and its ability to regulate crystal growth in vitro (19, 20). As in solution (19), LRAP has also been shown to assemble into nanospheres on fluorooapatite (21) and on surfactant-coated gold surfaces (22). Furthermore, it has been shown (23) that non-phosphorylated LRAP [LRAP(−P)] and recombinant full-length human amelogenin (rH174) have the same capacity to bind calcium (i.e. four to six calcium ions per molecule), although the calcium affinity constant for LRAP was greater than that for the full-length amelogenin. Based on similarities of structure and behavior, LRAP has allowed us to investigate the potential role of specific amino-acid domains of amelogenin and phosphorylation in protein self-assembly using dynamic light scattering (DLS) and transmission electron microscopy (TEM). Such studies have illustrated potentially important differences in the self-assembly behavior of phosphorylated LRAP [(LRAP(+P)] and LRAP(−P) (19).

The aim of the present study was to extend these recent findings using small angle X-ray scattering (SAXS), to investigate further the role of phosphorylation in LRAP self-assembly, in the presence and absence of calcium, through comparative studies of LRAP(+P) and LRAP(−P) forms of LRAP (Fig. 1).
Material and methods
Preparation of amelogenin peptides
LRAP(+P) and LRAP(−P) forms of porcine LRAP (56 amino acids) were synthesized commercially (NEO Peptide, Cambridge, MA, USA) and purified as previously described (24). Lyophilized peptides were weighed and dissolved in distilled deionized water at room temperature to yield stock solutions of 5–6 mg ml\(^{-1}\) of peptide. The solutions were kept at room temperature for 30 min and then stored at 4°C for 20 min just before use.

SAXS measurements
Aliquots of peptides were adjusted to a pH of ~7.4 with small amounts of KOH and HCl to obtain final concentrations of 2 mg ml\(^{-1}\) (0.31 mM) and 5 mg ml\(^{-1}\) (0.76 mM) of LRAP (total volume 70 \(\mu\)l). In selected experiments, calcium chloride was added to peptide solutions before adjustment of pH to yield final concentrations of 0.76–2.5 mM calcium (total volume = 70 \(\mu\)l). Hence, on a molar basis, the calcium to protein ratios ranged from 1.0 to 8.1. All sample preparations were carried out at room temperature.

Solution X-ray scattering experiments were carried out at the National Synchrotron Light Source at Brookhaven National Laboratory, on beamline X9 (25). The X-ray wavelength was 0.918 Å and the sample-detector distance was 3.4 m. The sample holder was a 0.9-mm-diameter quartz capillary tube that was open at both ends to allow continuous flow of the sample to avoid X-ray damage. Each experiment. The two-dimensional images acquired on a PILATUS 300K detector (Dectris, Baden, Switzerland) were averaged into one-dimensional scattering curves and then water scattering was subtracted using the pyXS software developed at the beamline. Solution X-ray scattering data were further analyzed using Primus software (26). The data consisted of the scattering vector, \(s\) [defined as \(s = 4\pi\sin(\theta)/\lambda\), and the corresponding intensity, \(I(s)\)]. As noted in the Results section, Guinier and Kratky plots were used to analyze the data. Guinier analyses consisted of the plotting of \(\log I(s)\) vs. \(s^2\) at very low \(s\) values and were used to determine the radius of gyration, \(R_g\), and the extrapolated intensity at the zero scattering angle, \(I_0\). With \(R_g\) being the mass distribution of the macromolecule around its center of gravity (27). Kratky analyses were carried out by plotting \(I(s) \times s^2\) vs. \(s\) and provided information on peptide folding, as described later (28). The number of molecules per particle was estimated using the following formula: \(N = (V_S \times N_A \times \delta_p)/F_W\); where, \(V_S = (4\pi R_g^3)/3\), the density of the protein \(\delta_p = 1.44 \text{g cm}^{-3}\) (3), \(N_A\) is the Avogadro constant, and \(F_W = 6537.52 \text{Da}\).

Fig. 1. Amino-acid sequences of the full-length porcine amelogenin (P173) and the phosphorylated (+P) and non-phosphorylated (−P) leucine-rich amelogenin peptide (LRAP). Note that LRAP is composed exclusively of the N- and C-terminal domains (shown in bold type) of the full-length molecule.

Results
Size of particles formed
Small angle X-ray scattering enables determination of the molecular size, shape, and conformational change of a protein molecule in solution. As shown in Fig. 2, the scattering generally exhibited fairly good linear dependence [\(\log I(s)\) vs. \(s^2\)] at low \(s\), indicating little to no bulk aggregation and allowing further data processing. As shown in Table 1, Guinier plots revealed that the \(R_g\) was 48.0 ± 0.05 Å for LRAP(−P) but only 25.0 ± 0.50 Å for LRAP(+P) (both at 2 mg ml\(^{-1}\), 13°C, pH 7.4), in the absence of added calcium. The \(R_g\) values for each peptide were very similar when the peptide concentration was increased to 5 mg ml\(^{-1}\), with an \(R_g\) of 46.3 ± 0.02 Å for LRAP(−P) and an \(R_g\) of 27.4 ± 0.08 Å for LRAP(+P), under the same experimental conditions. The addition of 2.5 mM calcium to 2 mg ml\(^{-1}\) solutions of peptide, however, induced a significant increase in the \(R_g\) for LRAP(+P) to 43.9 ± 0.05 Å, but it had relatively little effect on the \(R_g\) of LRAP(−P), with a value of 46.2 ± 0.03 Å being determined (Table 1). Similar results were obtained for 5 mg ml\(^{-1}\) peptide solutions containing 2.5 mM calcium, yielding \(R_g\) values for LRAP(+P) and LRAP(−P) of 46.4 ± 0.06 Å and 53.9 ± 0.06 Å, respectively. As also shown in Table 1, using peptide concentrations of 5 mg ml\(^{-1}\), increasing concentrations of calcium added to LRAP(−P) solutions resulted in a progressive, but slight, increase in the \(R_g\) values, whereas increased calcium concentrations resulted in marked concentration-dependent increases in the \(R_g\) values for LRAP(+P).

Increased \(I_0\) values per unit peptide concentration (c), \(I_0/c\) (Table 1), calculated for the LRAP(+P) samples, however, were consistent with peptide self-assembly (29), as particle sizes \(R_g\) increased by an overall factor of around two upon the addition of higher concentrations of calcium. In contrast, as clearly seen in 5 mg ml\(^{-1}\) samples, a much smaller increase in \(I_0/c\) was observed for LRAP(−P) upon the addition of calcium. Similar increases in the number of molecules per particle were also observed (Table 1). Overall, under comparable conditions, \(I_0/c\) values were larger for LRAP(−P) than for LRAP(+P) and corresponded to a somewhat greater number of molecules per particle.

Determination of conformational changes
Kratky plots [\(I(s) \times s^2\) vs. \(s\)] of SAXS data present characteristic and distinctly different shapes for globular fol-
dependent upon the concentration of calcium added (Fig. 3C). The addition of 0.76 mM calcium induced the formation of a very slight peak, suggesting that the peptide remained mostly in the unfolded state with the appearance of some globular features; upon the addition of 1.5 mM calcium the peak appeared more pronounced, indicating an increase in peptide folding. With the addition of 2.5 mM calcium, the peak of the Kratky plot for LRAP(+P) became even more pronounced and began to resemble that of the non-phosphorylated LRAP(-P) peptide, although the curves remained somewhat distinct at higher values of s (Fig. 3A–D).

**Discussion**

Leucine-rich amelogenin peptide, like amelogenin, is a phosphorylated protein that is secreted with a single phosphate group on serine 16 (33). Previous studies from our laboratory have shown that this single phosphate group has a major influence on the properties of amelogenin. In particular, in comparison with their non-phosphorylated (recombinant or synthetic) counterparts, phosphorylated (native and synthetic) forms of full-length porcine amelogenin (P173) (7), truncated porcine amelogenin P148 (8), full-length LRAP(+P) (19), and a truncated form of LRAP [LRAP(+P,–CT)] (20) all exhibit the capacity to stabilize amorphous calcium phosphate (ACP) effectively [i.e. prevent its transformation to crystalline hydroxyapatite (HA)], under experimental conditions designed to support the spontaneous formation of calcium phosphates in vitro. In sharp contrast to these findings, non-phosphorylated forms of full-length amelogenin and full-length LRAP were shown to guide the formation of ordered bundles of apaticite crystals (5, 8, 19). This latter behavior has been attributed to the ability of full-length amelogenin (6, 7, 15, 34) and LRAP (19) to form higher-order chain-like structures under specified conditions of pH. The presence of calcium has also previously been shown to enhance the formation of chain-like structures of recombinant full-length mouse rM179 (5) and human rH174 (35), amelogenin, and LRAP (19). Notably, as shown in the latter study, particle sizes and the formation of chain-like structures were enhanced to a greater degree for the phosphorylated form of LRAP. This conclusion was based on DLS and TEM observations. Our present SAXS findings support this conclusion and further demonstrate that the presence of calcium has a profound effect on the size and conformation of LRAP(+P) and little effect on those characteristics of LRAP(-P). This effect is caused by the presence of a single phosphate group on serine 16.

Guinier (Table 1) and Kratky (Fig. 3) analyses revealed further assembly and enhanced folding of LRAP(+P) upon the addition of calcium (Fig. 3A–C). Further assembly with added calcium is indicated (Table 1) by marked increases in Rg values, in estimates of the number of molecules per particle, and in Iq/c values. However, it should be noted that Iq values are affected by factors other than particle size (i.e. Rg), such as particle shape and particle density, in the proportion...
in vitro calcium phosphate precipitation behavior and for the effects that these peptides have on explanation for the differences observed in assembly conditions. Additional studies will be needed to provide presence of the highest calcium concentration studied at Fig. 3, however, differences in Kratky plots for calcium concentration (Fig. 3A,B,D). Moreover, as demonstrated here (Fig. 3), LRAP(+P) exhibits a more unfolded structure than LRAP(−P), as LRAP(−P) is inherently more tightly folded and key calcium-binding sites are concealed. Furthermore, observed differences in folding, and the tendency for LRAP(+P) to form less folded structures in the absence and presence of calcium, may also explain why phosphorylated LRAP(+P) is much more effective in stabilizing ACP nanoparticles and preventing the transformation of ACP into HA. The more open structure and the calcium-induced folding of LRAP(+P), in the presence of calcium and phosphate under mineralizing conditions, could lead to a more effective sequestration of forming ACP nanoparticles that prevents the transformation of ACP to HA. The fact that LRAP(−P) has been shown to stabilize ACP only transiently (19) is consistent with the present findings and this developing hypothesis on the effect of phosphorylation on protein conformation and mineralization. However, it is important to consider that previous findings also show that LRAP(−P) (19), like the full-length non-phosphorylated amelogenins rM179 (5) and rP172 (8), has the capacity to regulate the formation of ordered arrays of apatitic crystals by guiding the alignment, fusion, and subsequent transformation of the initially formed ACP nanoparticles (8). This capability

### Table 1

<table>
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<tr>
<th>Peptide concentration</th>
<th>[CaCl₂] (mM)</th>
<th>Guinier</th>
<th>Number of molecules per particle</th>
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<tr>
<td></td>
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<td>$R_g$ (Å)</td>
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<td>2 mg ml⁻¹</td>
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$R_g$ is the root-mean-square radius of gyration, $I_o$ is the intensity at zero scattering angle, $I_o/c$ is the $I_o$ value per unit peptide concentration. $R_g$, $I_o/c$ values, suggest that LRAP(+P) remains some-

$Io/c \propto (\rho_{\text{peptide}} - \rho_{\text{solvent}})^2 \times V_{\text{particle}}$, where $\rho$ indicates (electron) density and $V_{\text{particle}}$ indicates the volume of a single particle (27). Although our present analyses do not take potential differences in these latter factors into consideration, LRAP(+P) and LRAP(−P) were found to behave quite differently in the absence and presence of calcium, particularly with respect to folding. In contrast to that seen with LRAP(+P), as observed previously using circular dichroism (36), LRAP(−P) does not undergo significant conformational change upon the addition of calcium (Fig. 3A,B,D). Moreover, as demonstrated here (Fig. 3), LRAP(+P) exhibits a more unfolded structure than LRAP(−P), particularly in the absence and presence of lower concentrations of calcium. It appears that in the presence of calcium, unfolded LRAP(+P) molecules assemble to form more globular-like structures and the process is primarily triggered by calcium concentration (Table 1 and Fig. 3). As seen in Fig. 3, however, differences in Kratky plots for LRAP(+P) and LRAP(−P), which persist even in the presence of the highest calcium concentration studied at high $s$ values, suggest that LRAP(+P) somewhat more unfolded than LRAP(−P) under these conditions. Additional studies will be needed to provide insight into other potential differences in LRAP(+P) and LRAP(−P) self-assembly, as briefly noted above.

The remarkable conformational difference between LRAP(+P) and LRAP(−P) shown here offers a possible explanation for the differences observed in assembly behavior and for the effects that these peptides have on calcium phosphate precipitation in vitro. Changes in the folding of LRAP(+P), induced by the addition of calcium, may result from favorable calcium interactions with specific peptide sites that are exposed in the less-folded LRAP(+P) molecules. Such interactions lead to an enhancement of protein–protein interactions and to the formation of higher-order anisotropic chain-like structures (19). Hence, based on our present findings, it is concluded that the presence of the single phosphate group in LRAP induces functionally important conformational changes in the protein structure that favor the formation of higher-order protein assemblies. Accordingly, specific structure-changing calcium interactions that take place with LRAP(+P) do not occur with the non-phosphorylated LRAP(−P), as LRAP(−P) is inherently more tightly folded and key calcium-binding sites are concealed. Furthermore, observed differences in folding, and the tendency for LRAP(+P) to form less folded structures in the absence and presence of calcium, may also explain why phosphorylated LRAP(+P) is much more effective in stabilizing ACP nanoparticles and preventing the transformation of ACP into HA. The more open structure and the calcium-induced folding of LRAP(+P), in the presence of calcium and phosphate under mineralizing conditions, could lead to a more effective sequestration of forming ACP nanoparticles that prevents the transformation of ACP to HA. The fact that LRAP(−P) has been shown to stabilize ACP only transiently (19) is consistent with the present findings and this developing hypothesis on the effect of phosphorylation on protein conformation and mineralization. However, it is important to consider that previous findings also show that LRAP(−P) (19), like the full-length non-phosphorylated amelogenins rM179 (5) and rP172 (8), has the capacity to regulate the formation of ordered arrays of apatitic crystals by guiding the alignment, fusion, and subsequent transformation of the initially formed ACP nanoparticles (8). This capability
was found to depend on the presence of the hydrophilic C terminus in both amelogenin and LRAP (20). Based on these collective findings, additional processes [e.g. proteolysis or dephosphorylation, as we have recently discussed (19)] may be involved in vivo to trigger the subsequent transformation of initially formed ACP particles, as found in the early stages of developing enamel (37). We propose that initially formed ACP mineral in developing enamel is stabilized by native phosphorylated amelogenins that guide the accumulation and linear arrangement of amorphous nanoparticles which serve as precursors to enamel crystallites.

In conclusion, we have shown that the single phosphate group in LRAP(+P) induces functionally important conformational changes, particularly with respect to calcium interactions. Although further studies are needed, the present findings suggest that phosphorylation may also influence amelogenin conformation and subsequent protein-mineral interactions during the early stages of amelogenesis.

Acknowledgement – This work was supported by grant DE-016376 (to H.C.M) from the National Institute of Dental and Craniofacial Research. Use of beamline X9 and the National Synchrotron Light Source, Brookhaven National Laboratory, was supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences, under contract no. DE-AC02-98CH10886.

Conflicts of interest – The authors declare that there are no conflicts of interest with respect to this manuscript.

References


