Amelogenin self-assembles to form an extracellular protein matrix, which serves as a template for the continuously growing enamel apatite crystals. To gain further insight into the molecular mechanism of amelogenin nanosphere formation, we manipulated the interactions between amelogenin monomers by altering pH, temperature, and protein concentration to create isolated metastable amelogenin oligomers. Recombinant porcine amelogenins (rP172 and rP148) and three different mutants containing only a single tryptophan (Trp161, Trp45, and Trp25) were used. Dynamic light scattering and fluorescence studies demonstrated that oligomers were metastable and in constant equilibrium with monomers. Stable oligomers with an average hydrodynamic radius ($R_h$) of 7.5 nm were observed at pH 5.5 between 4 and 10 mg ml$^{-1}$. We did not find any evidence of a significant increase in folding upon self-association of the monomers into oligomers, indicating that they are disordered. Fluorescence experiments with single tryptophan amelogenins revealed that upon oligomerization the C terminus of amelogenin (around residue Trp161) is exposed at the surface of the oligomers, whereas the N-terminal region around Trp25 and Trp45 is involved in protein-protein interaction. The truncated rP148 formed similar but smaller oligomers, suggesting that the C terminus is not critical for amelogenin oligomerization. We propose a model for nanosphere formation via oligomers, and we predict that nanospheres will break up to form oligomers in mildly acidic environments via histidine protonation. We further suggest that oligomeric structures might be functional components during maturation of enamel apatite.

Tooth enamel formation follows the general organic matrix-mediated biomineralization process in which the extracellular matrix components regulate the nucleation, growth, and organization of the forming mineral (1). Amelogenin accounts for ~90% of the protein content of the developing enamel matrix, and micrographs have revealed that it may exist in the form of self-assembled nanochains of nanospheres (2). Its primary function is to form a matrix that helps to guide the growth of highly anisotropic hydroxyapatite nanorods (3). The resulting picture is of a hierarchical structure built in a "bottom-up" fashion that begins with the creation of small oligomers and ends as a macroscale scaffold.

Several different forms of amelogenin monomers (4), nanospheres (2, 5), and nanochains (6, 7) have been isolated and studied extensively in vitro, but oligomeric forms have received relatively little attention. One recent study revealed that altering ionic strength by adding chloride salts could cause amelogenin monomers to assemble via their N and C termini (8). A previous study suggested the formation of oligomers as necessary precursors to nanosphere formation as small protein subunits were observed to link nanospheres together when viewed using transmission electron microscopy (7). Recent work also confirmed the formation of "small particles" with an average hydrodynamic radius between 4 and 8 nm just prior to nanosphere-nanosphere assembly, but a distinction between nanospheres and oligomers was not made (9).

The concept of proteins forming stable oligomers prior to assembling into much larger constructs is well established. In various protein systems, such as the amyloid protein Aβ40 (10, 11), Syrian hamster prion protein (12), hydrophobin SC3 (13), and tobacco mosaic virus (14), a metastable oligomeric form has been identified as forming the “building blocks” for or being a necessary precursor to the formation of larger organized protein structures.

With regard to secondary structure, amelogenin has been shown recently (4, 15) to belong to a growing class of proteins known as intrinsically disordered proteins (IDPs) (16). This disorder is most prominent when amelogenin is forced into a monomeric state, which can be easily achieved by dissolving the protein in acidic conditions. Once assembled as nanospheres, a slightly greater magnitude of β-sheet character can be observed in the conformation of amelogenin (17). What has not been elucidated is whether this increased level of folding is critical for nanosphere formation and whether it is a consequence of assembly or a consequence of other parameters, such as temperature and pH. Several studies have previously shown that

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3 The abbreviations used are: IDP, intrinsically disordered protein; $R_h$, hydrodynamic radius; DLS, dynamic light scattering.
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IDPs can undergo significant conformational alteration upon dimerization and subsequent oligomerization (18, 19).

The aim of this study was to highlight the importance of the substructures of amelogenin nanospheres and to elucidate the mechanism of amelogenin assembly. Herein, by observing early stages of amelogenin self-assembly under mildly acidic pH conditions, we studied any conformational changes that occurred due to IDP-IDP interactions. By defining the in vitro conditions under which stable oligomers can form, we studied the first interactions between amelogenin monomers. Several in vitro studies in recent years (7–9, 20, 21) and one in vivo study (22) have hinted at the presence of subnanosphere assemblies of amelogenin that may assist in chain propagation and/or exist as building blocks of the nanospheres. In this study, we investigated the existence of amelogenin “oligomers” by identifying conditions under which they could be isolated and studying their size, assembly properties, and the molecular interactions that lead to their formation. Furthermore, by creating single tryptophan amelogenins that behaved precisely in the same way as the wild type, we were able to identify changes in interactions at the N and C termini under three of the four primary conditions of assembly (monomers, oligomers, and nanospheres).

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Recombinant porcine amelogenin (rP172) was expressed in Escherichia coli strain BL21-codon plus (DE3-RP, Agilent Technologies, Inc., Santa Clara, CA) and precipitated by 20% ammonium sulfate (23). To synthesize rP148, a previously cloned pig amelogenin cDNA construct encoding P173 was used as the template for polymerase chain reaction-mediated site-directed mutagenesis. The construct encoding P173 was used as the template for polymerase chain reaction-mediated site-directed mutagenesis.

Fluorescence—The full-length recombinant porcine amelogenin (rP172) was expressed in

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rP172(W161), rP172(W25), and rP172(W45), respectively, corresponding to the single tryptophan remaining in the sequence. As each mutant has two point mutations, the mutation was introduced in two steps. Initially, a rP172(W25Y) plasmid was obtained by PCR amplification using overlapping oligonucleotide primers containing the desired point mutation, which is TAT instead of TGG at the center of the oligonucleotide primers to mutate tryptophan to tyrosine at the 25th amino acid position. Later, the rP172(W25Y) plasmid was used as a template, and PCR amplification was done using appropriate oligonucleotide primers to replace tryptophan with tyrosine at the 45th amino acid position to get the rP172(W25Y, W45Y) plasmid. A similar procedure was followed to obtain the rP172(W45Y, W161Y) plasmid and the rP172(W25Y, W161Y) plasmid. Dynamic light scattering (DLS) studies of the Trp mutants revealed that there were no significant differences (from the wild-type rP172) in the level of self-assembly at pH 5.5 (oligomers) and pH 8 (nanospheres) (supplemental Fig. S1).

Sample Preparation for DLS, Circular Dichroism (CD), and Fluorescence—The temperature, pH, and protein concentration were all varied as detailed under “Results.” rP172 samples were prepared by first dissolving the lyophilized protein in cold water to an estimated concentration of 10–20 mg/ml, creating a stock solution. The stock was incubated at 4 °C for at least 2 days. The sample was then centrifuged at 10,000 × g for 1 h at 4 °C to remove any remaining undissolved aggregates. The top 90% of the solution was then removed, and the concentration was checked on a NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Wilmington, DE) using an experimentally calculated molar extinction coefficient of 17,144 l/mmol·cm⁻¹. The molar extinction coefficient of rP148 was experimentally calculated to be 12,945 ± 406 l/mmol·cm⁻¹. Samples prepared at specific pH conditions were prepared from aliquots of the stock solution by mixing with sodium acetate buffer (25 mM, pH 5.5) at 4 °C.

Dynamic Light Scattering—Dynamic light scattering of rP172 was performed using a Wyatt DynaPro Nanostar DLS instrument (Wyatt Technology, Santa Barbara, CA). Regularization fits of the data were plotted with percent mass as the y axis value. This is an important attribute to note as presenting DLS data using percent intensity as the y axis value can give higher hydrodynamic radius (R_h) values (relative to using the percent mass value) in heterogeneous systems. For room temperature experiments, the temperature was set to 22 °C, whereas variable temperature experiments were performed between 5 and 37 °C using a ramp rate of 0.1 °C·min⁻¹. The data were analyzed using
Dynamics 7.0 software. The dynamic light scattering data were produced by the program performing a regularization fit using the Dynals (Wyatt Technology Corp.) algorithm on the resultant autocorrelation functions. A Rayleigh sphere model was used for the analysis, meaning that the hydrodynamic radii calculated were sphere-equivalent radii. By measuring the fluctuations in the laser light intensity scattered by the sample, the instrument is able to detect the speed (diffusion coefficient) at which the particles are moving through the medium. This value is converted to the hydrodynamic radius using the Stokes-Einstein relation.

**Determination of Molecular Mass Based on Uversky Equation**—The hydrodynamic radii of the protein particles measured with DLS were further converted into a molar mass using Equation 1, which was determined by Uversky (25) and designed for pre-molten globular (PMG) proteins,

\[
\log(R_{\text{S,PMG}}^\text{NU}) = -(0.239 \pm 0.055) + (0.403 \pm 0.012) \cdot \log(M)
\]

(Eq. 1)

where \( R_{\text{S,PMG}}^\text{NU} \) indicates the hydrodynamic radius of a natively unfolded pre-molten globular protein and \( M \) is the molar mass of the protein.

**Fluorescence Spectroscopy and Anisotropy**—Fluorescence experiments were carried out using a PTI QuantaMaster QM-4SE instrument (Photon Technology International, Birmingham, NJ). An excitation wavelength of 295 nm was used in all experiments. For steady state fluorescence experiments, the window size of the excitation and emission shutters was set to 3 nm. A 10-nm window size was used for the anisotropy experiments. The fluorescence data were analyzed using FeliX32 software, and peaks were fitted using Lorentz peak functions with FindGraph v2.1 software. The concentration of amelogenin for titration experiments was 0.25 mg/ml, and peaks were fitted using Lorentz peak functions with FindGraph v2.1 software. The fluorescence data were analyzed using Jasco Spectra Manager.

**CD Spectropolarimetry**—A Jasco J-815 circular dichroism spectropolarimeter was used to perform CD experiments. Samples with a concentration of 0.2 mg/ml were placed inside a 1-mm quartz cuvette for CD analysis, whereas more concentrated samples (1–5 mg/ml) were sandwiched between two quartz plates with an approximate path length of 0.01 mm. Four acquisitions were taken and averaged at a scan rate of 50 nm/min using a digital integration time of 4 s. CD spectra were analyzed using Jasco Spectra Manager.

**Nuclear Magnetic Resonance (NMR) Spectroscopy**—Liquid-state NMR experiments were performed on two \(^{13}\text{C}\),\(^{15}\text{N}\)-uniformly labeled rP172 samples with uniformly labeled \(^{13}\text{C},^{15}\text{N}\) rP172 prepared as described elsewhere (4). The first was a 75 \( \mu \)M sample in unbuffered deionized distilled water, pH 3.8 (monomeric state) (4), and the second was a 386 \( \mu \)M sample (final concentration determined by UV-visible spectrophotometry) in 25 mM sodium acetate buffer, pH 5.5 prepared as described elsewhere in this report. Samples were 300 \( \mu \)l in volume and were loaded in 5-mm symmetrical Shigemi tubes (Shigemi, Inc., Allison Park, PA) to enhance sensitivity. \(^{13}\text{C},^{15}\text{N}\) heteronuclear single quantum coherence and one-dimensional \(^{1}\text{H}\) NMR experiments were performed on both samples using an ultrashield Bruker US2 AVANCE spectrometer (21.14-tesla superconducting magnet; Larmor frequencies of 900.264 MHz for \(^{1}\text{H}\), 226.370 MHz for \(^{13}\text{C}\), and 91.22 MHz for \(^{15}\text{N}\)) outfitted with a 5-mm HXY triple resonance Bruker cryoprobe. The \(^{1}\text{H}\) 90° pulse was 9.35 \( \mu \)s, and a spectral width of 12 ppm was used. Water suppression was achieved using gradient excitation sculpting.

**RESULTS**

We identified conditions under which metastable rP172 and rP148 amelogenin oligomers can form. Our strategy was to change parameters, such as pH, temperature, and protein concentration, to understand the driving forces behind assembly and disassembly. Furthermore, by creating single tryptophan amelogenins, we were able to study which parts of the amelogenin monomer were associated upon oligomerization.

**Metastable Oligomers Formed at pH 5.5**—We sought to investigate the influence of pH on the local conformation and self-assembly of the protein by fluorescence measurements. To do this, we probed the environment around the three Trp residues of amelogenin (Scheme 1) over a range of pH values (3.4–9.4) by illuminating the protein at a 295-nm excitation wavelength and following their shift in the emission maximum (Fig. 1). The most striking observation from steady state intrinsic tryptophan fluorescence was a sharp blue shift of 7–8 nm in the emission maximum that occurred between pH 5.8 and 6.1 and was concomitant with visible aggregation. A similar observation has been made previously in the case of human amelogenin (26), although the narrow range in which the shift occurred was not noted. Prior to the large blue shift at pH 5.8, we observed a smaller gradual blue shift of 3 nm between pH 3.5 and 5.5 (Fig. 1). Physically, this revealed a transition from an extended to a more compact structure and that the environment around tryptophan residues was gradually becoming more hydrophobic, implying that the monomers present under acidic conditions were assembling as the pH was increased.

Between pH 5.8 and 9, the fluorescence emission maximum was observed to be consistently below 340 nm, whereas at higher pH values, the fluorescence emission maximum red shifted back to 350 nm. The low emission wavelength observed between pH 5.8 and 9 was consistent with the knowledge that amelogenin exists in the forms of nanospheres and nanochains under these conditions (5, 9, 27), although it was not possible to create nanospheres directly from oligomers by increasing the pH. The oligomers tended to aggregate as the pH increased above pH 6. As pH was increased to 10, a red shift was observed as the negative charge on individual amelogenin monomers destabilized the nanospheres.

The change in fluorescence anisotropy of both rP172 and rP148 between pH 3.5 and 5.5 was studied to give some insight into the cause of the blue shift (Fig. 2). Fluorescence anisotropy measures the rate of rotation of a fluorescent molecule by mea-
suring how polarized light is depolarized by the molecule under analysis (28). An increase in anisotropy indicates that the fluorescent molecule is rotating more slowly, and a slower rotational correlation time is caused by an increase in the size of the fluorescent molecule given a constant temperature and viscosity. The anisotropy value increased from 0.054 to 0.081 between pH 3.5 and 5.5 at 5 mg/mL, indicating that rP172 monomers were beginning to self-associate at higher pH (i.e. pH 5.5) (Fig. 2). Importantly, the increase in anisotropy between pH 3.5 and 5.5 was observed in samples in which the sodium ion concentration was corrected to 25 mM, indicating that the oligomerization was, in this case, due to the change in pH and not ionic strength. It is equally important to note that lowering the buffer concentration to very low levels (\(<25\text{ mM}\)) will prevent oligomers from forming even at pH 5.5. This is because lowering the ionic strength increases the Debye screening length (29), preventing hydrophobic regions from coming into close contact.

Fluorescence anisotropy of rP148 at 5 mg/mL at pH 3.5, 4.5, and 5.5 also revealed that oligomerization began to occur as pH was increased (Fig. 2), although the increase in anisotropy was significantly less pronounced than that seen with rP172. The data shown represent samples in which the sodium ion concentration was controlled at 25 mM.

**Identification and Characterization of Amelogenin Oligomers**

**FIGURE 1.** Plot of wavelength emission maxima of rP172 over range of pH values between 3.4 and 9.4. The blue shift in emission wavelength was most apparent at around pH 6, although a small shift occurred at lower pH (3.5–5.8). The concentration of amelogenin at the end of the titration was 0.25 mg/mL. Individual experiments (labeled as triangles) were performed at an amelogenin concentration of 0.3 mg/mL. Schematics of the monomeric, oligomeric, and nanosphere forms are indicated at the relevant pH. Experiments were performed at 22 °C.

**FIGURE 2.** Fluorescence anisotropy measurements revealed pH dependence on oligomer formation of both rP172 and rP148. Both rP172 and rP148 self-assembled at pH 5.5, although the rP148 oligomers appeared to be smaller. These experiments were performed using sodium acetate buffer (25 mM) with the sodium ion concentration adjusted to 25 mM by addition of the appropriate amount of sodium chloride.
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regard to forming oligomers. However, the creation of oligomers was also dependent on both protein concentration and temperature when the pH of the system was in the region of interest (pH 4.5–5.8). The $R_\text{H}$ values of rP172 oligomers were measured at 22 °C at pH 5.5 and at a range of protein concentrations from 0.25 to 10 mg·ml$^{-1}$ (5–510 μM). Fig. 3 shows how the average hydrodynamic radius of the major rP172 structural “unit” (i.e. monomers or oligomers) decreased with increasing dilution at 22 °C from around 7.5 nm at 10 mg·ml$^{-1}$ to around 4.0 nm at 0.5 mg·ml$^{-1}$. A clear plateau in $R_\text{H}$ centered at around 7.2 nm could be observed between 10 and 4 mg·ml$^{-1}$. Importantly, the same kind of trend could be applied to fluorescence anisotropy data on the same samples (supplemental Fig. S2), verifying that the decrease in $R_\text{H}$ observed by DLS during dilution was not an artifact of raising the translational diffusion coefficient due to decreasing hydrodynamic interactions with decreasing concentration (30, 31). The rotational diffusion coefficient is not significantly affected in this concentration range (32).

Oligomers Are in Fast Equilibrium with Monomers and Dimers—Examining the effect of temperature on oligomerization at a defined concentration (2.5 mg·ml$^{-1}$ or 127 μM, pH 5.3) suggested that the oligomers are in dynamic equilibrium with their monomeric counterparts. A concentration of 2.5 mg·ml$^{-1}$ was used because it was below the plateau region observed during the experiments that determined the effect of concentration on oligomerization. It was therefore possible to observe an increase in oligomer size with temperature change. The slightly lower pH helped to avoid aggregation at high temperature (>30 °C) that disrupted the study of the reversibility of the formation of oligomers. Upon heating from 5 to 37 °C, DLS revealed an increase in the hydrodynamic radius from $3.9 \pm 0.1$ to $4.9 \pm 0.2$ nm (Fig. 4). The overall intensity of scattered light (measured by the detector as a voltage) increased from 0.27 to 0.34 V, which was concomitant with the increase in $R_\text{H}$ and indicated a lack of large scale aggregation upon heating (supplemental Fig. S3). The changes in size and scattering were completely reversible with no obvious hysteresis observed when using a ramp rate of 0.1 °C·min$^{-1}$.

Unstructured Oligomers Can Be Formed via Hydrophobic N-terminally Mediated Assembly—Three independent techniques were used to investigate amelogenin-amelogenin self-association. CD experiments were performed in parallel with DLS and anisotropy experiments to elucidate the origin of folding within rP172 upon self-association. NMR experiments were carried out to gain information about the rotational mobility of the backbone, whereas single tryptophan fluorescence provided localized information about the environment of different parts of the amelogenin molecule.

The relative state of folding of each rP172 sample was measured by comparing the ellipticity at 200 nm with that at 222 nm (17). Increasing the temperature from 5 to 37 °C increased the order within the structure regardless of pH, whereas altering the pH at one specific temperature increased the observable order less significantly. Fig. 5, a–c, show the CD spectra at pH 3.5, 5.5, and 8 at 5 and 37 °C and 0.2 mg·ml$^{-1}$. Fig. 5d shows the 200:222 nm ellipticity ratios under the same conditions. That temperature induced folding even under dilute and acidic conditions when amelogenin is monomeric (4) indicates that this increase in structural order is primarily intramolecular and not a result of protein-protein binding. Increasing the pH from pH 3.5 to 5.5 to 8 caused a slight increase in conformational order at low concentration (0.2 mg·ml$^{-1}$) (Fig. 5). This weak conformational change was not observed at high concentration (5 mg·ml$^{-1}$) (supplemental Fig. S4), although this may have been due to protein adsorption onto the quartz plates between which the high concentration amelogenin samples were studied. Again, as with the change in temperature at pH 3.5, any change in structural order was intramolecular and purely due to a pH change rather than a direct result of the interaction between amelogenin molecules.
To obtain more information on the nature of the assembled complex, we performed high field NMR experiments on isotopically labeled rP172 in the monomeric form and in the oligomeric form at pH 5.5. Due to inhomogeneous broadening effects, we were unable to obtain multidimensional NMR spectra (${^{15}\text{N}}-{^{1}\text{H}}$ heteronuclear single quantum coherence) of rP172 at pH 5.5, indicating that the backbone motion of these protein molecules is very limited within these assemblies. This was verified by comparing the one-dimensional $^{1}$H NMR spectra obtained for both the monomeric and assembled forms (Fig. 6). Here, it is clear that significant line width broadening and signal reduction are present at pH 5.5 but not at pH 3.8. These spectral effects arise from intermediate time exchange broadening that results from slow polypeptide backbone motions within the
large molecular weight pH 5.5 assemblies. In contrast, the monomeric pH 3.8 sample exhibits greater mobility. This result, coupled with the absence of heteronuclear single quantum coherence signals for the pH 5.5 rP172 sample, suggests that most if not all of the sequence regions within rP172 assemblies are motionally restricted and experience slower tumbling compared with the monomeric form.

The porcine amelogenin contains three tryptophan residues; two of them are located at the N terminus (Trp25 and Trp45), and the other is close to the C terminus (Trp161) of the sequence (Scheme 1). Accordingly, we created three single tryptophan amelogenins for study with fluorescence. These are denoted as rP172(W25), rP172(W45), and rP172(W161) to indicate the presence of the unmodified tryptophan.

Fluorescence spectroscopy (λmax = 295 nm) of the Trp mutants was performed to probe the local changes in environment of each tryptophan residue in the sequence. Fig. 7 shows the change in emission maximum (λmax) of each Trp mutant and wild-type rP172 with change in pH. The pH values were chosen so the amelogenin would be either monomeric (pH 3.5), oligomeric (pH 5.5), or in nanosphere form (pH 8).

The blue shift that was observed for the rP172(W161) variant in an oligomeric form (pH 5.5) relative to the monomeric form (pH 3.5) was not significant. In this case, the emission maximum was shifted from 348.5 nm in monomeric form to 347.6 nm in oligomeric form. However, for the rP172(W25) and rP172(W45) variants, a significant blue shift was observed as the protein transforms from monomer to oligomers (Δλmax = 4 nm) and then to nanospheres (Δλmax = 12 nm). These results indicate that the N-terminal tryptophan residues undergo a shift from a solvent-exposed hydrophilic environment in the monomeric form to a hydrophobic environment upon oligomerization and nanosphere formation.

**DISCUSSION**

In biology, there are many examples of self-assembling systems that utilize an oligomeric species either as a building block or a compulsory intermediate for the formation of larger structures (10–14). The unique ability of amelogenin to form monodisperse nanospheres containing between 50 and 100 monomers under certain conditions led us to question whether there is an underlying substructure that is critical to the formation and function of the nanosphere. As previous studies in this area have suggested a potential role of oligomers in nanosphere and matrix formation (7–9), we followed this up by studying the size and folding response of oligomers to pH, temperature, and protein concentration changes.

The fluorescence anisotropy data at pH 3.5, 4.5, and 5.5 immediately revealed that amelogenin monomers were associating at the higher pH level (Fig. 2). The fact that an increase in anisotropy could be observed in the C-terminally truncated amelogenin rP148 demonstrated that the C terminus was not critical in causing oligomerization under the conditions used.

Dynamic light scattering was not used to follow changes in hydrodynamic radii with pH in the acidic range (pH <5 for rP172 and pH <6 for rP148) as charged particles can artificially appear too small. This is because the translational diffusion coefficient of the charged particles is increased by repulsive Coulomb interactions causing them to diffuse faster (33–36). The rotational diffusion coefficient is not significantly affected at the low ionic strengths and particle concentrations in the range used in this study (37, 38). The effect on translational diffusion coefficient is most noticeable at high protein concentration and low ionic strength.

Ultimately, the evidence of oligomerization at around pH 5.5 was not entirely unexpected as the protein visibly aggregated when the pH was raised above 5.8, indicating that self-association was promoted as the pH became more basic. Amelogenin is monomeric at acidic pH (e.g. pH 3.5) as it becomes positively charged, and this electrostatic charge causes repulsion between monomers as observed with DLS. As the pH is increased above approximately pH 4, the eight acidic residues (most of which are located near the C terminus) become deprotonated. At this stage, amelogenin still holds a positive charge as its 14 histidine residues will still be protonated. As the pH is increased, deprotonation of histidine residues causes the molecule to have an overall neutral charge and lose electrostatic stabilization. Indeed, the fluorescence intensity of rP172(W45) at pH 3.5 and 5.5 was low in comparison with that of rP172(W25) (Fig. 7, a and b) but higher at pH 8 (Fig. 7c). This change might in part be attributed to solvent quenching, but it is more likely a consequence of the deprotonation of His47 and His48 as protonated histidine residues have been shown to have a large effect on the quenching of tryptophan relative to deprotonated histidine (39). The pKₐ of His has been reported to be 6.6 (40), although this value can range from around pH 2.5 to 9 (41). Accordingly, the midpoint of the large shift in fluorescence occurs around pH 6.1, which is close to the pKₐ value of histidine and is concomitant with visible aggregation of the protein. ζ-Potential studies on recombinant human amelogenin have shown that there is no charge on the protein at around pH 6.8 and that a small positive charge on the protein cannot cause dispersion as effectively as a small negative charge (26).

As the concentration was increased at pH 5.5, the hydrodynamic radii of the amelogenin particles also increased. Remark-
ably, $R_H$ remained at around 7–7.5 nm as rP172 was diluted from 10 down to 4 mg/ml. Below 4 mg/ml, $R_H$ began to drop with decreasing concentration. The lack of decrease in $R_H$ during dilution from 10 to 4 mg/ml indicated that the 7.5-nm oligomers were stable. Once the concentration dropped too low (below 4 mg/ml), the oligomers began to break up into smaller units. The error margin of the "plateau radius" within each experiment was fairly low with an average standard deviation of 2–3%. Critically, fluorescence anisotropy measurements revealed a similar plateau (supplemental Fig. S2), demonstrating that the DLS plateau was not caused by the effect of Coulomb interactions on the translational diffusion coefficient as described earlier. Because amelogenin under acidic conditions is classified as a premolten globule subclass of IDPs, the status of the particles observed was analyzed using the equation reported by Uversky (25) for this group of proteins (see Equation 1 under "Experimental Procedures"). The values of 3.1, 5.5, and 7.2 nm correspond to a monomer, tetramer, and octamer of amelogenin, respectively.

Such a plateau with increasing protein concentration has been observed previously in the case of the fusion protein peptibody A (42). In that case, the authors could explain the plateau by observing that oligomerization must have terminated at a specific oligomer size (heptamers). This was supported by the fact that a decrease in polydispersity was observed as the plateau was reached. In the case of amelogenin oligomers, the polydispersity did not decrease as the plateau was reached, implying that a stable equilibrium was formed above 4 mg/ml. The C-terminal tryptophan only shifted a further 2.7 nm relative to its maximum at pH 5.5. The intensities of both N-terminal tryptophan residues were much greater than that of Trp161. The increase in intensity of Trp45 implies that the nearby histidine residues became deprotonated. d, a chart summarizing the wavelength shifts of rP172(W161) (black circles), rP172(W45) (open circles), rP172(W25) (crosses), and rP172 (diamonds) under the three different pH conditions tested. rP172 fluorescence was recorded as rP172 contains all three tryptophan residues.
mass of 200 kDa (10 monomers in the case of rP172). In agreement with our present finding, an atomic force microscopy-based protocol was used recently to determine the size of amelogenin oligomers that were stabilized on a mica surface at pH 8. By combining atomic force microscopy height measurements and a DLS-based calibration curve (43), it was reported that oligomers contain between 7 and 14 monomers and are decameric on average.

That the average oligomer radius decreased significantly with a decrease in temperature indicates that oligomer formation is an endothermic process as higher temperatures increase the thermal energy of each amelogenin monomer, allowing them to overcome the activation energy required for self-association. This has been noted previously using calorimetry (17). These results corroborate what many authors have noted previously: amelogenin is constantly in equilibrium with the monomeric species even at pH values that promote nanosphere or nanochain formation (44–46).

It is important to note that our experiments were performed under low ionic strength conditions (25 mM) and in the absence of divalent cations. Buchko et al. (8) have previously demonstrated oligomerization via the C termini with increasing ionic strength. As expected from those findings, when we increased the ionic strength of the oligomer suspensions at pH 5.5 with CaCl$_2$, the hydrodynamic radius increased up to 8.5 nm ([CaCl$_2$] = 25 mM) (supplemental Fig. S6). Using Equation 1, this radius corresponds to a molar mass of 240 kDa, which is larger than the oligomers that are formed primarily via hydrophobic interactions.

The most striking observation from the single tryptophan amelogenin fluorescence study was that upon forming nanospheres the N-terminal tryptophans (Trp$^{25}$ and Trp$^{45}$) underwent a blue shift of 12–13 nm relative to the monomeric condition, whereas the C-terminal tryptophan (Trp$^{161}$) did not shift as significantly. This shows that that some regions of the 45 N-terminal amino acids of amelogenin are buried in a relatively hydrophobic pocket in a nanosphere. The relative lack of shift in emission maximum of the Trp$^{161}$ residue indicated that the C terminus was more exposed to the aqueous environment. From this observation, we can infer that the C-terminal region is not as heavily involved in assembling the nanosphere as the N-terminal region under the low ionic strength conditions tested. The N-terminal region has been identified previously as a region involved with amelogenin self-association and was termed "Domain A" (47).

Previous studies have suggested that amelogenin oligomers may play a functional role in the formation of nanospheres and higher order self-assembled structures (7–9, 20, 21). By studying the oligomers in isolation at pH 5.5 and by separating the roles of the C terminus and N terminus in assembly, it was possible to improve the model for the self-assembly of amelogenin.

The model published by Fincham et al. (20) suggested that 4–5-nm “intermediates” were formed prior to nanosphere formation, but it was suggested that the N and C termini interacted in forming these intermediates. The model proposed by Du et al. (7) also suggested that oligomers associate to form nanospheres but proposed that the C termini were permanently exposed either on the outer surface of the nanosphere or facing water channels within the nanosphere. Here, via fluorescence experiments on single tryptophan variants of amelogenin, we were able to confirm that the C terminus is indeed in a more solvent-exposed environment in both the oligomeric and nanosphere forms. The model proposed by Du et al. (7) considered amelogenin monomers to be globular, but recent work has shown that amelogenin is an intrinsically disordered protein when in the monomeric form (4, 15). In this study, we experimentally confirmed that this disorder is maintained during the initial assembly into oligomers and also within nanospheres. The plateau in the hydrodynamic radius with increasing protein concentration indicated that as more monomers are added to the oligomer the growth becomes self-limiting, reaching a particular size range. Oligomers in this size range contain around eight monomers on average, whereas the polydispersity indicates that there is a broad distribution around this average size. This would occur as a result of the increased burial and resultant inaccessibility of the N-terminal domains in larger oligomers. At pH 5.5, the growth of oligomers via N-terminally mediated assembly is prevented beyond a critical average size of oligomer. At this pH, the central region of the protein contains a large number of protonated histidine residues that are well conserved between species (48). For example, 13 of 14 histidine residues are conserved between porcine and human amelogenin. Increasing the pH to physiological values deprotonates many of these histidine residues (26), which could then allow the hydrophobic residues in the central region to begin to take part in the assembly of oligomers to create nanospheres.

Based on our experimental data, we have updated our previous model for assembly. Fig. 8a shows a graphic of the improved model of self-assembly of amelogenin. It is based on the model described by Du et al. (7) except the monomers are no longer considered globular, and the positioning of the C terminus in a more aqueous environment has now been confirmed experimentally. More importantly, we have separated the interactions that cause monomers to assemble into oligomers and those that cause oligomers to bind and form nanospheres. Specifically, we propose that oligomer formation is primarily N-terminally mediated (potentially N terminus to N terminus), whereas nanosphere formation is mainly regulated by hydrophobic interactions via the central portion that in turn are controlled by histidine protonation. In the model, we have shown the entire N-terminal region as being in the core of the oligomer for simplicity. However, our data only provide information about the environments around residues 25 and 45 in the N-terminal region, so it is possible, for example, that the tip of the N terminus is not buried in a hydrophobic pocket.

That nanospheres break up to form oligomers in mildly acidic media may have relevance in vivo. As carbonated hydroxyapatite enamel crystals grow, several protons are released per unit cell created (49). pH fluctuations between mildly acidic and neutral were observed during the maturation stages of enamel formation. The acidic values reported included pH 5.8–6 in the case of calves (50) and pH 6.2–6.9 in...
the case of rats (51). This would create a localized mildly acidic environment, ensuring that any amelogenin in the vicinity would become slightly protonated. Not only an acidic environment but also positively charged surfaces can stabilize amelogenin oligomers (21). This combination would result in the formation of a nanosphere. The “unstructured” nature of the oligomers makes them ideal for interaction with various targets, such as apatite (52, 53), enamelin (53, 54), ameloblastin (55), and the cell surface (56). Fluorescence experiments with single tryptophan amelogenins revealed that whereas the C terminus of amelogenin (specifically residue Trp161) resides in an aqueous environment even when assembled into oligomers or nanospheres the N-terminal region around Trp36 and Trp45 moves into a more hydrophobic environment as soon as monomers begin to assemble into oligomers. When nanospheres are formed at pH 8, the local environment of the two N-terminal tryptophans becomes even more hydrophobic (λmax ≈ 333 nm). From these observations, it was determined that oligomer formation is mediated by hydrophobic interactions in the N-terminal region, and it was inferred that nanospheres could be formed via weak hydrophobic interactions between exposed central region sequences when the multiple histidine residues in that section were deprotonated (for example at pH 8). Future work in this area will focus on the energy of interactions between monomers, oligomers, and nanospheres and the role of histidine residues in the central region of amelogenin.

CONCLUSIONS

In conclusion, we identified and fully characterized oligomeric species of amelogenin, demonstrating the relative lack of increase in conformational order upon the initial self-assembly event. We demonstrated that oligomers were metastable and in constant equilibrium with monomers. The lack of increase in size above a particular concentration (≈4 mg·mL⁻¹) indicated that as oligomers increase in size the tendency to assemble becomes impaired and is ultimately self-limiting. This finding has important biological implications as the protein concentration in the enamel extracellular “fluid” is above 5 mg·mL⁻¹ and yet oligomers may remain metastable. NMR experiments revealed that amelogenin molecules within pH 5.5 assembles were inhomogeneously broadened and experienced slower tumbling and limited backbone motion compared with the monomeric state.

Regarding folding, we found no evidence of any significant increase in conformational order upon self-association to oligomers. The “unstructured” nature of the oligomers makes them ideal for interaction with various targets, such as apatite (52, 53), enamelin (53, 54), ameloblastin (55), and the cell surface (56). Fluorescence experiments with single tryptophan amelogenins revealed that whereas the C terminus of amelogenin (specifically residue Trp161) resides in an aqueous environment even when assembled into oligomers or nanospheres the N-terminal region around Trp36 and Trp45 moves into a more hydrophobic environment as soon as monomers begin to assemble into oligomers. When nanospheres are formed at pH 8, the local environment of the two N-terminal tryptophans becomes even more hydrophobic (λmax ≈ 333 nm). From these observations, it was determined that oligomer formation is mediated by hydrophobic interactions in the N-terminal region, and it was inferred that nanospheres could be formed via weak hydrophobic interactions between exposed central region sequences when the multiple histidine residues in that section were deprotonated (for example at pH 8). Future work in this area will focus on the energy of interactions between monomers, oligomers, and nanospheres and the role of histidine residues in the central region of amelogenin.

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