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ABSTRACT

The developing enamel matrix is a highly dynamic system mainly composed of the full-length amelogenin and its proteolytic cleavage products. In this study, size, zeta-potential, and the isoelectric points of nanoparticles of the recombinant full-length human amelogenin (rH174) and two proteolytic products (rH163 and rH146) were analyzed by dynamic light-scattering and electrokinetic measurements. We tested the hypothesis that zeta-potential may be used as a control parameter in directing the self-assembly of amelogenins. Extensive aggregation of amelogenin molecules with the particle size reaching about one micron occurred at a mildly acidic to neutral pH, and coincided with the red shift of the internal fluorescence. Zeta-potential was between ± 15 mV in the same pH range, indicating that amelogenin aggregation occurred when surface potentials were minimal. This suggests that electrostatic interactions may be another crucial factor, aside from hydrophobic interaction, in the aggregation and hierarchical assembly of spherical particles of amelogenins into supramolecular structures of a higher order.

KEY WORDS: amelogenin, dynamic light-scattering, isoelectric point, MMP-20, zeta-potential.

Zeta-potential and Particle Size Analysis of Human Amelogenins

INTRODUCTION

Dental enamel develops in a protein matrix-mediated process of mineralization known as amelogenesis (Garant, 2003). Self-assembly of this protein matrix, 90% of which is initially composed of various amelogenins, is expected to play a crucial role in the proper morphogenesis of this tissue. Understanding the physical and chemical conditions that govern the physiologically relevant self-assembly of amelogenin proteins may thus be an essential step in replicating this complex biological process *in vitro* and producing artificial structures that resemble enamel (Habelitz *et al.*, 2004, 2005; Uskoković *et al.*, 2008).

The growth of the mineral phase during amelogenesis occurs in parallel with the degradation of the protein matrix that guides the former. Consequently, attention must be paid to the proteolytic effects involved in the maturation of the enamel matrix (Bartlett and Simmer, 1999). Matrix-metalloprotease-20 (MMP-20) is known to be the first protease to hydrolyze amelogenin, and, unlike the more aggressive action of another major protease in the process, kallikrein-4, it cleaves amelogenin in a highly selective manner. The function of these specific enzymatic reactions and their products is not yet understood, but may be crucial for the proper assembly of the enamel matrix and its ability to direct apatite crystal growth. Therefore, we studied the ζ -potential of 3 recombinant human amelogenins: the full-length protein (rH174) and 2 recombinant MMP-20 cleavage products (rH163 and rH146), known to be the first 2 large peptides formed following the proteolytic digestion of rH174 (Zhu *et al.*, 2008). The ζ -potential of amelogenin in aqueous suspension and isoelectric points (pI) of the 3 amelogenin proteins were analyzed in context of the hypothesis that ζ -potential may be used as a control parameter in directing the self-assembly of recombinant human amelogenins.

MATERIALS & METHODS

The recombinant amelogenins rH174, rH163, and rH146 were expressed in BL21DE3 plysS *Escherichia coli* according to a procedure described previously (Li *et al.*, 2003). Their identity was confirmed in a mass spectrometric analysis (Voyager DE STR, MALDI-TOF; Applied Biosystems, Carlsbad, CA, USA). We prepared suspensions of 0.4 mg/mL amelogenins at 25°C by dissolving the lyophilized protein in a 20 mM Tris/HCl buffer at pH 2, with pH manually adjusted between the individual measurements by KOH or HCl. The measurements of the average particle size and ζ -potential of the aqueous suspensions of amelogenin were carried out on a Malvern Zetasizer Nano-ZS dynamic light-scattering (DLS) analyzer (Malvern Instruments Ltd., Malvern, Worcestershire, UK). Samples ($n > 3$ for each protein, including various batches) of 1 mL were

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Figure 1. Functions of the average particle size and ζ -potential on pH for the aqueous suspensions of rH174 (**a**), rH163 (**b**), and rH146 (**c**) ($N = 15, 6, 4$, respectively), including the amino acid sequences thereof (**d**). The graphs were made by overlapping the results of analyses of proteins derived from different recombinant batches, and each datapoint was obtained as an average of over 100 acquisitions. The bolded amino acids in the sequences of rH174, rH163, and rH146 represent the residues with titratable side-chains.

analyzed for particle size and ζ -potential in multiple runs, in a pH-increasing manner, with approximately 2 hrs required to cover the examined pH range. Each datapoint was the result of averaging over 100 acquisitions, each lasting about 10 sec. The particle sizes reported presented hydrodynamic diameters derived from the time-correlation function of the particle number density (Berne and Pecora, 2000). The pIs of the analyzed proteins were experimentally determined by interpolation of mean ζ -potential values at the zero value. The intrinsic fluorescence of rH174 was analyzed at 37°C with excitation wavelengths of 280 and 293 nm recorded in the range of 300–700 nm (Genesys V; Thermo-Scientific, Waltham, MA, USA). No significant difference in the spectra was observed at 280 or 293 nm excitation. We used atomic force microscopy (AFM, Nanoscope III; Digital Instruments, Santa Barbara, CA, USA) to characterize the protein aggregates morphologically. We

prepared samples for AFM analysis by pipetting 20 μ L of the buffered suspension onto a glass slide, incubating the specimen for 1 hr in a wet cell, washing the slide with a few droplets of water, and immediately drying it with canned air (He *et al.*, 2008). Tapping-mode AFM was applied with Si-tips with a radius of about 5 nm (Supersharpe, Nanosensors, Neuchâtel, Switzerland).

RESULTS

Plots showing the dependence of zeta-potential and particle size on pH were derived for all the analyzed proteins: rH174, rH163, and rH146 (Fig. 1). As pH of the suspension medium increased, ζ -potential dropped from more than +20 mV at pH < 4 to less than -20 mV at pH > 9. Zeta-potential range in the ± 15 mV zone corresponded well to the pH range at which the extensive aggregation

Table. Comparison of the Experimentally Determined pI Values for rH174, rH163, and rH146 and Those Estimated with Different Theoretical Models Based on pK_a Averaging over the Titratable Groups of the Peptide Chain *

Protein	Experimental	EMBL Palabra	Scripps	DTA				Sillero Rodwell
				Select	Select	Select	Select	
rH174	6.77 ± 0.27	6.57	6.53	7.05	7.05	6.56	6.96	6.56
rH163	6.97 ± 0.26	6.78	6.72	7.23	7.24	6.78	7.15	6.77
rH146	6.52 ± 0.62	8.09	7.40	7.94	7.91	8.12	7.92	7.62

The listed theoretical models can be respectively found at the following Web pages: <http://www.embl.de/cgi/piwrapper.pl>; <http://www.nihilnovus.com/Palabra.html>; <http://www.scripps.edu/~cdputnam/protcalc.html>; and <http://isoelectric.ovh.org>.

and destabilization of the amelogenin suspensions were observed.

A comparison is given between the experimentally determined values for pI of rH174, rH163, and rH146 and the theoretically estimated values (Table).

Using the density (0.78 g/cm³) and molecular weight of rH174 (19.8 kDa), we calculated the Perrin factor (number of monomers per particle of a given size) as equal to 1 for particles of 4 nm in diameter. Considering a possible compression that the soft-protein spheres are prone to undergo during aggregation, this number may be subject to uncertainty. As pH of the suspension medium increased above pH 3, the protein monomers transformed to aggregates with diameters of 20–40 nm. Only occasionally were such aggregates detected at a pH as low as 1.5. The transition from monomers to 20- to 40-nm-sized nanospheres was not as sharp and readily detectable as the subsequent transition from nanospheres to particles of 1 μ m or more in size at pH 4–5.

The intrinsic fluorescence spectra of rH174 suspensions at different pH values are shown in Fig. 2. At pH < 5, when the aggregation of the nanospheres was negligible, the peak is located at $\lambda = 350$ nm. At pH 6 to 8.5, the peak maximum shifted to a lower wavelength, $\lambda = 340$ –345 nm.

The AFM analyses confirmed the formation of particles larger than 100 nm at pH 5.5. Such particles were composed of nanospheres of about 30 nm in size (Fig. 3a). The likelihood of our observing the nanosphere aggregation into larger entities using AFM was very low. The prevalent structure observed was the nanosphere of 20–40 nm in diameter in the pH range of 3–10 (Fig. 3b). The images shown are in agreement with the increasing polydispersity index (PDI) following the aggregation of the nanospheres. PDI in the DLS measurements ranged from 0.25 for 20 nm-sized spheres to 0.8 for 1 μ m-sized ones, corresponding to the standard deviations of ± 10 nm and ± 0.9 μ m, respectively.

DISCUSSION

This study shows that ζ -potential is associated with the aggregation propensity of recombinant human amelogenins. DLS studies on amelogenin molecular aggregates were previously carried out on amelogenins from species other than human (Moradian-Oldak *et al.*, 1998, 2000; Petta *et al.*, 2006). It has previously reported that aggregation of amelogenin takes place in the vicinity of the calculated pI (Wiedemann-Bidlack *et al.*, 2007). In this study, the same aggregation effect was analyzed in relation to both experimentally determined and theoretically estimated pIs.

Figure 2. Intrinsic fluorescence spectra of aqueous suspensions of rH174 at different pHs ($\lambda = 280$ nm).

The full-length amelogenin is intrinsically positively charged in the acidic pH range, due to protonation of the amino group at the N-terminal (Pro/P), and the carboxyl group at the C-terminal (Asp/D in the case of rH174 and Pro/P in the case of rH163 and rH146) and the 13 other basic groups of the protein backbone belonging to the Arg/R, Lys/K, and Tyr/Y side-chains. Likewise, the intrinsic negative charge of the protein in the alkaline pH range is caused by the reverse dissociation effects, that is, deprotonation of the Pro/P residue at the N-terminal, and the carboxyl group at the C-terminal and the 21 acidic groups of the protein backbone belonging to the Asp/D, Glu/E, and His/H side-chains.

Zeta-potential of the particles was relatively low even at the highest value (~ 25 mV). With increasing the pH from the highly acidic values at which amelogenin monomers exist in the solution, the ζ -potential decreased, letting the hydrophobic interactions unfold and leading to the formation of nanospheres. At potentials falling below +15 mV, corresponding to pH of 4.2–4.7, the aggregation of nanospheres occurred. Zeta-potential of $\sim +15$ mV posed a critical boundary above which the electrostatic repulsion between individual protein molecules overcame the hydrophobic attraction between them, and thereby prevented the formation of nanospheric aggregates. Aggregation of protein was enhanced at the positive side of the ± 15 mV ζ -potential zone. Thus, as soon as charges became negative, nanoparticles re-dispersed, more easily than at positive values. Even at ζ -potential as low as -3 mV, in the case of rH174 and rH163, and -5 mV, in the case of rH146, particles of 40 nm and less were predominant in the suspension, indicating the presence of characteristic amelogenin nanospheres. The protein nanoparticles were thus more sensitive to negatively charged surfaces of the adjacent spheres, since they easily caused their repulsion and re-dispersion. This is consistent with the fact that biological entities are most often dispersed on the side of negative and relatively low values of ζ -potential (Riddick, 1968).

Figure 3. AFM images of rH174 aggregates dispersed in water and deposited on glass, at pHs 5.5 (**a**) and 7.6 (**b**).

Theoretical pI values for rH174 and rH163 fall within the measured pI range determined in this study, while the measured pI range of rH146 is significantly lower than the calculated values. The unknown secondary and tertiary structures of the analyzed proteins and the polydisperse nature of the particles in the vicinity of the pI contribute to the uncertainty in both estimation and measurement of the pI. The supposedly random distribution of surface-exposed residues within the polydisperse aggregates of nanospheres and their unstable suspension additionally spread the pH range in which pIs of individual particles are found. This all contributes to the fact that the highest statistical difference between measured pIs was found between those of rH174 and those of rH163: $P < 0.13$.

According to the model proposed by Snead (2003), amelogenin molecules assemble into nanospherical particles while having a U-shape, with both the hydrophilic C-terminal and TRAP sequence at the N-terminal exposed on the nanoparticle surface, which is supported by the observed quick processing of both terminals in the presence of MMP-20. Hence, a large sequence close to the middle of the rH174 sequence (residues 45-110), in which His is the only titratable residue (10 His residues lying in this range), would be buried within the particle, contributing significantly less to the protein charge. The effect of shielding of His residues ($pK_a = 6.1$) from the surface would, in this case, be offset by the surface exposure of the likewise mildly acidic 11-amino-acid portion at the C-terminal (theoretical pI = 6.5). Furthermore, to compensate for the contribution of the latter to the acidic pK_a of the surface, the TRAP sequence (residues 1-44), with its alkaline nature (theoretical pI = 8.8), must, to a certain extent, be exposed on the nanosphere surface.

The fluorescence spectra corroborated the correlation between results on the particle size and zeta-potential of the dispersed protein aggregates. The shift to lower wavelengths as the pH of the suspension of rH174 increased from 5 to 6 was explained by an increase in the hydrophobicity of the environment surrounding the aromatic rings (belonging to 3 Trp/W, 7 Tyr/Y, and 1 Phe/F residues of rH174) that produce the fluorescent effect. As the nanoparticles aggregated in this pH region,

more of the aromatic rings became buried within the protein agglomerates and thus exposed to a more hydrophobic surrounding, resulting in a downshift of the internal fluorescence (Barth and Zscherp, 2002).

Still, different theoretical models yield different results, and they are used here only as reference points that vaguely display the pH range at which the pI of the proteins should be expected to be found. As expected, the pI of rH163 was higher than that of rH174, since the mildly acidic 11-amino-acid portion ($pK_a \sim 6.5$) of the C-terminal was cleaved off the full-length molecule. The extent of the pI increase was partly offset by the exposure of the more acidic carboxyl group of the Pro residue at the C-terminal of rH163, as compared with the same group in the Asp residue at the C-terminal of rH174.

In addition to the effect of shielding of specific residues following the protein folding, in practically the entire pH range in which the ζ -potential measurements were conducted, the protein existed in the form of assemblies and aggregates, owing to its high content of Pro and Gln residues (42%), which are known to be involved in the facile formation of intermolecular hydrogen bonds and extended chains. Hence, the particulate nature of amelogenin in aqueous suspensions results in another type of partition of residues: those buried inside the nanospheres, with limited contribution to the ζ -potential, and those exposed on the surface, fully contributing thereto. In the case of rH146, the deviation of the experimental results from the theoretical expectations indicated that the surfaces of rH146 particles do not reflect the average content of its titratable residues. Furthermore, not only does amelogenin exhibit a conformational change following its assembly into nanospheres, but so also do the cleavage products following amelogenin digestion by MMP-20 (Lakshminarayanan *et al.*, 2007).

The typical formation of nanospherical, 20- to 40-nm-sized amelogenin particles in aqueous media is driven by hydrophobic forces, which exist as a result of the mildly amphiphilic nature of amelogenin—that is, the residues positioned in the vicinity of the C-terminal are more hydrophilic than the

rest of the molecule, whereas the internal, hydrophobic residues have a tendency to clump together. Immobilization of the micro-sized protein particles on the glass surface, as performed prior to AFM imaging, often resulted in re-dispersion of smaller, nanosized subunits, forcing us to conclude that there is only a weak attraction between the amelogenin nanospheres within larger, micron-sized aggregates. This observation is in agreement with the results of a previous study on the onset of amelogenin aggregation, in which analysis of the DLS data suggested the existence of larger entities, whereas the small-angle x-ray scattering (SAXS) measurements detected smaller particle sizes (Aichmayer *et al.*, 2005). The easily reversible transition between the micron-sized and nanosized particles through manipulation of the pH, as observed by DLS, is further evidence of the loose character of the large protein aggregates.

Finally, although the results of the study relate to a specific protein, amelogenin, the meaning of the correlations established between ζ -potential and particle-particle attraction could be potentially applied for self-assembling proteins in general. For example, enzyme-ligand binding is favored under conditions of electrostatic attraction between the two, which open the space for more subtle bonding effects (Wade *et al.*, 1998). As recently shown for amelogenin protein matrices, the first step in the merging of protein nanospheres, eventually leading to fibrous self-assembled morphologies, is conditioned by a narrow window of optimal surface charge attractions. Merging of amelogenin nanospheres into fibrous entities was observed predominantly under pH conditions at which amelogenins were oppositely charged (He *et al.*, 2008). Such an elongation of amelogenin assemblies through aggregation of nanospheres is expected to be involved in guiding the growth of high aspect-ratio apatite crystals of enamel. Consequently, ζ -potential may be used as a control parameter in replicating the assembly of amelogenins *in vitro*. While the electrostatic interactions are required for the first step, *i.e.*, the approaching of the assembling entities (*i.e.*, amelogenin nanospheres), weak physicochemical interactions involved in subtle molecular recognition effects subsequently emerge, constituting the decisive influence in “shaping” the assembled entities into their final forms.

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