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ABSTRACT

Amelogenin, the major protein of forming dental enamel, plays a crucial role in the biomineralization of this tissue. Amelogenin is soluble at low pH and self-assembles to form higher order structures at physiological pH. To understand the mechanisms of its assembly and interactions with calcium phosphate mineral, we conducted FTIR spectroscopy (FTIRS) studies of pH-triggered assembly of recombinant porcine amelogenin rP172 and its interactions with mature hydroxyapatite and apatitic mineral formed *in situ*. Analysis of our data indicated that rP172 at pH 3.0 exists in an unfolded disordered state, while increases in pH led to structural ordering, manifested by increases in intra- and intermolecular β -sheet structures and a decrease in random coil and β -turns. Amelogenin assembled at pH 7.2 was also found to contain large portions of extended intramolecular β -sheet and PPII. These FTIRS findings are consistent with those previously obtained with other techniques, thus verifying the validity of our experimental approach. Interestingly, interactions with mineral led to a reduction in protein structural organization. The findings obtained show that amelogenin has intrinsic structural flexibility to accommodate interactions with both forming and mature calcium phosphate mineral phases, providing new insights into the potential importance of amelogenin-mineral interactions in enamel biomineralization.

KEY WORDS: enamel, hydroxyapatite, protein self-assembly, protein-mineral interactions, biomineralization, intrinsically disordered protein.

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Structural Changes in Amelogenin upon Self-assembly and Mineral Interactions

INTRODUCTION

The highly ordered structural organization of enamel is controlled by extracellular matrix proteins during the secretory stage of amelogenesis. The organization of secretory enamel crystals into enamel rods is similar to that of mature enamel; however, at this stage, the organic matrix accounts for 20 to 25% w/w (Fukae *et al.*, 2007) of the tissue. During maturation, this organic matrix is degraded and enamel crystals thicken to yield a mineralized tissue that is > 95% mineral.

Amelogenin, the most abundant enamel protein, plays a major role in regulating mineral formation (Paine *et al.*, 2000; Gibson *et al.*, 2001). It consists of 3 domains: an N-terminal domain called tyrosine-rich amelogenin peptide (TRAP), containing a single phosphorylation site at Ser-16; a central domain enriched in Pro and Glu; and a hydrophilic C-terminus. TRAP and C-terminal domains play essential roles in amelogenin self-assembly (Paine *et al.*, 2000).

In solution, amelogenin is globally unfolded with large fractions of β -sheet and β -strand concentrated in the TRAP, while the central domain adopts an extended conformation rich in random coil, polyproline type II (PPII) helix, and β -turns (references in Margolis *et al.*, 2006; Delak *et al.*, 2009; Lakshminarayanan *et al.*, 2009). Amelogenin assembles into spherical or oblate-shaped aggregates, called nanospheres, 15 to 20 nm in diameter (Fincham *et al.*, 1994; Aichmayer *et al.*, 2010; Wiedemann-Bidlack *et al.*, 2007) via a multistep hierarchical process (Fang *et al.*, 2011a,b). Amelogenin exists in monomeric form at low pH and self-assembles at neutral and moderately basic pH values (Wiedemann-Bidlack *et al.*, 2007). Nanospheres organize into chains, which form three-dimensional networks at pH values close to the pH of the protein (Beniash *et al.*, 2005; Du *et al.*, 2005; Wiedemann-Bidlack *et al.*, 2007, 2011; Fang *et al.*, 2011b).

Amelogenin regulates calcium phosphate mineralization *in vitro* through cooperative interactions with forming mineral, leading to the formation of crystalline arrays similar to those in developing enamel (Beniash *et al.*, 2005; Kwak *et al.*, 2009; Deshpande *et al.*, 2010; Yang *et al.*, 2010; Wiedemann-Bidlack *et al.*, 2011). Notably, amelogenin transiently stabilizes amorphous calcium phosphate (ACP) *in vitro* (Kwak *et al.*, 2009; Yang *et al.*, 2010), which, combined with the finding of ACP in forming enamel (Diekwisch, 1998; Beniash *et al.*, 2009), suggests its role in regulating the mineral phase.

Although recent advances have been made toward an improved understanding of amelogenin-mineral interactions (Beniash *et al.*, 2005; Kwak *et al.*, 2009; Tarasevich *et al.*, 2009; Yang *et al.*, 2010; Fang *et al.*, 2011a), critical details are lacking. The studies presented here test the hypothesis that

Figure 1. Overlays of Amide I and Amide II regions of the normalized spectra of rP172 in solutions at pH 3.0 (—), pH 5.6 (---), and pH 7.2 (- · -) (A); and in the presence of hydroxyapatite (—) and forming mineral (- · -) (B). The spectrum of rP172 in PBS at pH 7.2 is also presented (B).

amelogenin conformational changes upon self-assembly differ from those associated with interactions with calcium phosphate mineral. Here, we utilize FTIRS to study the pH-triggered assembly of recombinant porcine amelogenin, rP172, and its interactions with calcium phosphate minerals. FTIRS is extremely sensitive to global conformational changes in proteins (Surewicz *et al.*, 1993; Wang *et al.*, 2010) and is uniquely suited to the study of structural changes in proteins upon self-assembly (Bouchard *et al.*, 2000; Wang *et al.*, 2010) and adsorption (Roach *et al.*, 2005; Elangovan *et al.*, 2007). While other techniques, such as NMR and x-ray diffraction, may provide much more detailed structural information, FTIR, unlike these other methods, can be used to study protein conformation even when proteins are in an aggregated state or adsorbed to surfaces. The results of this article advance our understanding of amelogenesis and will aid in the design of novel enamel regeneration approaches and improved restorative materials.

MATERIALS & METHODS

Protein Preparation

Recombinant porcine amelogenin (rP172) expressed in *E. coli* was produced as previously described (Simmer *et al.*, 1994). Protein stock solutions (pH ~ 3) were prepared as described in the Appendix.

FTIRS of rP172 in Solution

FTIR spectra of rP172 in DDW and in PBS were collected at different pH values in attenuated total reflection (ATR) mode, according to a previously described approach (Elangovan *et al.*, 2007) (see Appendix). Specific pH values were selected based on the variations in amelogenin aggregation along the pH spectrum, as described in the Appendix.

rP172 Binding to Hydroxyapatite (HA) and Mineral Formed *in situ*

Mineral-binding experiments with stoichiometric HA crystals (NIST, Gaithersburg, MD, USA) were conducted at pH 7.2, as described in Elangovan *et al.* (2007). To induce mineralization in the presence of rP172, we added CaCl_2 to 5 mg/mL solution of rP172 in PBS to establish a Ca^{2+} concentration of 2.5 mM and a phosphate concentration of 4 mM. Samples were incubated at 37°C for 16 hrs. The samples were studied by ATR-FTIRS (see Appendix for details).

FTIR Spectroscopic Analysis of rP172 Conformation

FTIR spectroscopic data collection and analyses of rP172 conformation were performed as described previously (Elangovan *et al.*, 2007) and in the Appendix.

RESULTS

Overall, FTIR spectral profiles of amelogenin at pH 3.0, 5.6, and 7.2 differed considerably (Fig. 1A), indicating that variations within this pH range induce significant conformational changes. However, the spectra of amelogenin at pH 8.0 and 7.2 were similar (Fig. 2C,D).

rP172 in DDW (pH ~ 3.0)

At pH 3.0, amelogenin remained in monomeric form, based on our DLS analysis. The FTIR spectrum of rP172 at pH 3.0 features a broad Amide I band with absorbance maxima around 1,630 cm^{-1} , 1,650 cm^{-1} , and 1,670 cm^{-1} , and a shoulder at ~1,620 cm^{-1} (Figs. 1A, 2A). The Amide I absorbance band contains 4 major sub-bands that were assigned as hydrated PPII-helix (1,619 cm^{-1}), random coil (1,644 cm^{-1}), and β -turn (1,662 cm^{-1} and 1,676 cm^{-1}) conformations (Fig. 2A, Table 1). Several smaller bands were also observed and were attributed to small percentages of β -sheet (1,628 cm^{-1} and 1,634 cm^{-1}) and β -turn or to the high-frequency split of anti-parallel β -sheets (1,686 cm^{-1}).

Table 1. Positions and Relative Areas of FTIR Absorption Peaks within Amide I Bands of rP172 at Different pH Values

Assignment	rP172 DDW		rP172 pH = 5.6		rP172 pH = 7.2		rP172 pH = 8.0	
	Peak Max (cm ⁻¹)	Peak Area (%)	Peak Max (cm ⁻¹)	Peak Area (%)	Peak Max (cm ⁻¹)	Peak Area (%)	Peak Max (cm ⁻¹)	Peak Area (%)
Aggregated β -strands with intermolecular h-bonds					1,613	23	1,613	29
Hydrated PPII	1,619	23	1,620	39	1,620	18	1,620	21
Intramolecular β -sheet	1,628	3	1,634	20	1,632	14	1,632	9
	1,634	8						
Random coil	1,644	24	1,648	10	1,645	12	1,645	10
					1,651	9	1,649	7
β -turn/ β_{10} helix	1,662	27	1,663	22	1,663	8	1,659	9
					1,670	11	1,669	8
Intramolecular β -sheet/ β -turn	1,676	13	1,679	9	1,682	5	1,679	6
	1,686	4						

Peak assignments are based on literature reports (see Appendix).

rP172 at pH 5.6

At pH 5.6, the Amide I band narrowed, with a well-developed peak formed around 1,630 cm⁻¹ and a long shoulder in a higher frequency portion of the band (Fig. 1A). Five peak maxima were identified (Fig. 2B) and assigned to hydrated PPII-helix (1,620 cm⁻¹), β -sheet (1,634 cm⁻¹), random coil (1,648 cm⁻¹), and β -turn (1,663 cm⁻¹ and 1,679 cm⁻¹) (Table 1 and Appendix). Although individual peaks identified at pH 5.6 are similar to those seen at pH 3.0, their relative contributions to the overall spectra changed significantly (Fig. 2B, Table 1). Specifically, the proportions of random coil and β -turn decreased notably with the increase in pH, while the amounts of hydrated PPII and pleated β -sheet increased.

rP172 at pH 7.2

At pH 7.2, the rP172 Amide I band features a sharp maximum ~1,620 cm⁻¹, with a broad shoulder in the high-frequency region (Fig. 1A). Peak-fitting analyses revealed 2 major peaks, comprising ~50% of the total Amide I band area (Table 1, Fig. 2C). One band with a maximum at 1,621 cm⁻¹ was assigned to the hydrated PPII structure, while the second band (1,613 cm⁻¹) was identified as an extended intermolecular hydrogen-bound β -sheet (Table 1, Appendix). Smaller bands at 1,632 and 1,648 cm⁻¹ were identified as pleated intramolecular β -sheet and random coil, respectively. Finally, peaks at 1,667 cm⁻¹ and 1,682 cm⁻¹ were attributed to β -turns.

Overall, the increase to pH 7.2 led to a low-frequency shift of Amide I absorbance intensity. These changes are due to an increase in intermolecular β -sheet and pleated intramolecular β -sheet structures and a significant decrease in random coil and β -turns (Table 1).

rP172 at pH 8.0

The shape of the Amide I band was very close to that observed at pH 7.2 (Fig. 2D). Peak analyses revealed that the contributions of individual peaks were similar to those at pH 7.2.

However, some differences were observed. Specifically, the contributions of low-frequency peaks assigned to aggregated β -strands and PPII-helices (Table 1) were higher at pH 8.0, while contributions of intramolecular pleated β -sheets were lower at this pH value.

rP172 in the Presence of HA

Based on published adsorption parameters, HA was fully covered by amelogenin, with 21-30% of the protein added being adsorbed (Nagadome and Margolis, 2002; Bouropoulos and Moradian-Oldak, 2003). The shape of the Amide I absorbance band of rP172 incubated with HA differed significantly from that of rP172 alone at pH 7.2 (Fig. 1B); the former band was broader, with a maximum at ~1,650 cm⁻¹ and shoulders at ~1,625 cm⁻¹ and ~1,665 cm⁻¹. FTIR analyses revealed a number of distinguishable peaks (Fig. 2D, Table 2, Appendix), corresponding to pleated β -sheet (1,628 cm⁻¹ and 1,634 cm⁻¹), random coil (1,654 and 1,642 cm⁻¹), and β -turns (1,671 cm⁻¹ and 1,686 cm⁻¹) structures. Analyses also revealed 2 large peaks (1,613 cm⁻¹ and 1,621 cm⁻¹), identified as intermolecular β -sheet and hydrated PPII-helix, respectively. The fraction of these peaks was less than those in rP172 spectra at pH 7.2 without mineral. Hence, interactions with mineral led to structural changes, manifested by an increase in random coil and β -turns, and a decrease in β -strand, β -sheet, and hydrated PPII-helix.

rP172 in the Presence of Forming Calcium Phosphate Crystals

Although the overall shape of the rP172 Amide I band in the presence of forming calcium phosphate mineral was similar to the spectrum obtained for rP172 incubated with stoichiometric HA (Fig. 1B), peak-fitting analyses revealed some differences. Specifically, the fraction of intramolecular β -sheet (1,635 cm⁻¹) was significantly higher in spectra from rP172 in the presence of forming calcium phosphate crystals than in the spectra of rP172 incubated with HA (Fig. 2E, Table 2). Similarly, the

Figure 2. FTIR spectra and individual fitted components of rP172 at pH 3.0 (A), pH 5.6 (B), pH 7.2 (C), and pH 8.0 (D), in the presence of hydroxyapatite (E) and forming mineral (F). Maximum wavenumbers for each fitted component are marked.

random coil fraction ($1,653\text{ cm}^{-1}$) was higher (Table 2). Furthermore, the fraction of aggregated β -strands with intermolecular h-bonds ($1,613\text{ cm}^{-1}$) was lower in the spectra of rP172 with forming calcium phosphate crystals (Table 2).

DISCUSSION

Analysis of our data indicates that, at pH 3.0, amelogenin is a globally unstructured protein containing large fractions of random coil, β -turn, and PPII-helices, with very little β -sheet and α -helical structures. These results are in agreement with those of

reports showing that monomeric amelogenin adopts an extended globally unstructured form in equilibrium with PPII (Delak *et al.*, 2009; Lakshminarayanan *et al.*, 2009). An increase in pH, however, dramatically affects rP172 conformation. At pH 5.6, there is a significant increase in β -sheet and reduction in random coil, compared with pH 3.0, suggesting an increase in protein structural organization. Other reports have shown amelogenin oligomerization and nanosphere formation at pH 5.6, although these oligomers and nanospheres are less organized than at pH values between 7.2 and 8.0 (Bromley *et al.*, 2011; Fang *et al.*, 2011b). Hydrated PPII helix was the dominant structure from

Table 2. Positions and Relative Areas of FTIR Absorption Peaks within Amide I Bands of rP172 in Mineral Binding Experiments

Assignment	rP172 with HA		rP172 with Forming Mineral	
	Peak Max (cm ⁻¹)	Peak Area (%)	Peak Max (cm ⁻¹)	Peak Area (%)
Aggregated β -strands with intermolecular h-bonds	1,613	20	1,610	13
Hydrated PPII	1,621	13	1,622	13
Intramolecular β -sheet	1,628	4		
	1,634	6	1,635	24
Random coil	1,642	10		
	1,654	22	1,653	25
β -turn/ 3_{10} helix	1,671	21	1,669	19
β -turn/intramolecular β -sheet	1,686	5	1,682	6

pH 3.0 to 8.0, despite variations in degrees of assembly. Analysis of these data indicates that amelogenin contains a relatively stable extended PPII helical domain, whereas other areas undergo significant pH-dependent conformational changes. These data are in good agreement with findings obtained by other approaches (references in Margolis *et al.*, 2006; Delak *et al.*, 2009; Lakshminarayanan *et al.*, 2009), and provide further evidence that changes in pH cause structural re-arrangements in amelogenin. Our recent observations (Fang *et al.*, 2011b) suggested that these conformational changes might be related to protein-protein interactions during higher order assembly of amelogenin oligomers.

Major structural changes were revealed at pH 7.2. At this pH, amelogenin assembles into highly organized oligomers and nanospheres that bind together to form branched chains (Wiedemann-Bidlack *et al.*, 2007, 2011; Fang *et al.*, 2011a,b). The most prominent conformational change associated with this pH is the appearance of a large fraction of intramolecular β -sheet structures, consistent with prior CD findings (Lakshminarayanan *et al.*, 2009) obtained in the presence of calcium. Another prominent spectrum feature corresponds to hydrated PPII helix. Together, these 2 peaks comprise ~40% of the Amide I band, suggesting that rP172 exists in an extended open conformation. The intramolecular β -strand is also a prominent feature of amyloid fibrils and their intermediate structures. The Amide I band profiles of amelogenin assemblies at pH 7.2 and amyloid fibrils are remarkably similar, featuring a prominent low-frequency peak and a higher-frequency shoulder [see Wang *et al.* (2010) and references therein]. Furthermore, it has been reported that amyloid fibril assembly occurs *via* unfolded or partially folded intermediates, containing large portions of PPII and random coil domains (Booth *et al.*, 1997; Wang *et al.*, 2010). Interestingly, our recent cryoEM studies demonstrated that amelogenin assembly occurs in a stepwise hierarchical process *via* oligomeric intermediates (Fang *et al.*, 2011a,b). Hence, as for the assembly of amyloid fibrils, we hypothesize that rP172 assembly is, in part, driven by the formation of intramolecular hydrogen bonds between extended unordered regions of the protein, leading to the formation of tightly bound intrafibrillar β -strands. Although at both pH 8.0 and 7.2, amelogenin forms oligomers which assemble into nanospheres, at pH 7.2 the

nanospheres further assemble into branched chains. The similarities in the rP172 spectra at these pH values indicate that major conformational changes are associated with protein oligomerization and nanosphere formation, while aggregation of nanospheres into chains does not cause major conformational changes.

Our FTIR analyses of amelogenin in the presence of mineral provide a unique glimpse into the mechanisms of protein-mineral interactions under physiological pH. Comparisons of spectra of amelogenin alone and with added HA at pH 7.2 indicate that rP172 undergoes significant conformational changes upon interactions with mineral. Specifically, proportions of random coil and β -turns increase in the presence of HA, while the fractions of intermolecular β -strands and PPII decrease, indicating a reduction in protein structural organization. Similar changes upon mineral interactions were previously found with salivary proline-rich protein 1 (PRP1) (Elangovan *et al.*, 2007), which, like amelogenin, is a relatively unstructured protein containing a large fraction of PPII-helix. These structural changes suggest that, as reported for PRP1 (Moreno *et al.*, 1991; Elangovan *et al.*, 2007), interactions of amelogenin with mineral are entropically driven and associated with a loss of hydration and the breaking of intramolecular bonds. Finally, the decrease in intermolecular aggregated β -strands might suggest the breaking of protein-protein interactions upon mineral binding. Hence, our results are in agreement with those of recent studies showing that amelogenin nanospheres disaggregate upon binding to fluorapatite surfaces (Tarasevich *et al.*, 2009; Chen *et al.*, 2011).

Spectra of rP172 incubated with stoichiometric HA, however, differ from those obtained in the presence of forming mineral. Specifically, no significant fraction of anhydrous/Ca-bound PPII protein structure was detected when mineral was formed in the presence of rP172. Analyses revealed an increase in beta-sheet in comparison with rP172 alone and rP172 incubated with HA. Importantly, observed differences in conformational changes induced by forming mineral in comparison with those caused by interactions with mature HA may reflect differences in the nature of the initial mineral phase formed *in situ* and/or differences in the organization of amelogenin *via* cooperative interactions with the forming mineral phase (Beniash *et al.*, 2005; Fang *et al.*, 2011a). ACP is the first mineral phase

formed in the presence of rP172 *in vitro* (Kwak et al., 2009; Yang et al., 2010), as well as during enamel deposition *in vivo* (Beniash et al., 2009). Furthermore, we have recently shown that amelogenin can interact with calcium phosphate pre-nucleation clusters (Fang et al., 2011a) and ACP nanoparticles (Kwak et al., 2009), and can organize them into parallel arrays prior to crystallization. Collectively, these results suggest different modes of amelogenin interactions with forming vs. mature calcium phosphate mineral.

In conclusion, we have shown that rP172 undergoes structural ordering upon pH-triggered self-assembly, while interactions with mineral lead to significant conformational changes and a reduction in protein structural organization. Our findings further show that amelogenin has intrinsic structural flexibility to accommodate specific interactions with both forming and mature calcium phosphate mineral phases, providing new insights into the potential importance of amelogenin-mineral interactions in enamel biomineralization.

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