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ABSTRACT

Amelogenin is one of the key protein constituents responsible for the exquisite organization of the calcium phosphate crystals in enamel. Amelogenin forms into nanospheres in solution, while its association with hydroxyapatite is also essential to enamel development. Structural information of full-length amelogenin in either of these physiologically important forms has the potential to provide mechanistic information; however, these data are limited because of the difficulty of determining the structure of large protein complexes and proteins bound to surfaces. To obtain structural insights into amelogenin during these early stages of enamel development, we used a lysine-specific ^{13}C -, ^{15}N -labeled sample of murine amelogenin to provide insight into the structure of the hydroxyapatite (HAP)-binding domains of the protein. A combination of one- and two-dimensional solid-state NMR experiments was used to obtain molecular-level insights into the secondary structure and dynamics of full-length amelogenin within a nanosphere-gel and on the surface of HAP. Regions of amelogenin that appear to be primarily random coil in the nanosphere-gel adopt a β -strand structure and are less mobile with HAP binding, indicative of a structural switch upon binding that may be important in the role of amelogenin in enamel development.

KEY WORDS: solid-state NMR, biomineralization, enamel, amelogenin, structural characterization, nanosphere.

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Mineral Association Changes the Secondary Structure and Dynamics of Murine Amelogenin

INTRODUCTION

Amelogenin, an ~22-kDa hydrophobic protein, is believed to play an essential role in enamel crystal nucleation and growth (Simmer and Fincham, 1995). Single-point mutations result in improper enamel formation, confirming its essential role (Ravassipour *et al.*, 2000); however, a mechanistic understanding of this role in successful or failed enamel formation has been elusive because of the limited structural data for amelogenin. The solution secondary and tertiary structure of the full-length protein has been studied under monomeric, low pH conditions and suggests a largely unstructured, intrinsically disordered protein, with possible structural changes as physiological pH is approached and amelogenin self-assembles into dimers, trimers, and larger oligomers (Buchko *et al.*, 2008; Delak *et al.*, 2009; Zhang *et al.*, 2011; Beniash *et al.*, 2012).

At physiological pH, macroscopic structural investigations in solution have shown that amelogenin self-assembles to form nanospheres 20 nm in diameter, consisting of 20 to 100 monomers (Moradian-Oldak *et al.*, 2000). These further assemble into nanosphere chains that are sensitive to pH and the phosphorylation state of Ser16 (Wiedemann-Bidlack *et al.*, 2011), and at higher concentrations into nanosphere-gels (Wen *et al.*, 1999). Nanospheres are also observed *in vivo*, suggesting an important role in enamel development (Fincham *et al.*, 1995). Packing of the monomers within the nanosphere has recently been studied by cryo-EM, revealing a double-ring barrel structure composed of 6 amelogenin dimers stabilized through C-terminal interactions (Fang *et al.*, 2011). Finer, molecular-level details of the monomer structure within the nanosphere have not yet been determined. It was also recently shown that amelogenin monomers, and not nanospheres, promoted the formation of needle-like, parallel arrays of calcium phosphate structures similar to those found in enamel (Beniash *et al.*, 2005). Additionally, AFM studies suggest that amelogenin monomers dissociate from nanospheres to interact with fluoroapatite (Tarasevich *et al.*, 2009a). These observations indicate that understanding the structure of amelogenin within the nanosphere and adsorbed onto hydroxyapatite (HAP) is important in the development of a mechanistic understanding of enamel growth. Unfortunately, structural studies have been difficult because of challenges in investigating large-molecular-weight self-assembled complexes or surface-immobilized proteins.

Solid-state NMR (SSNMR) spectroscopy is uniquely suited to the study of the structure of immobilized proteins, as demonstrated by dipolar recoupling studies that determined the structure and orientation of the amelogenin splice-variant LRAP bound to HAP (Shaw and Ferris, 2008; Masica *et al.*, 2011; Lu *et al.*, 2013). To date, studies of biomineralization proteins have been limited to small proteins (<~60 residues). In this work, we implemented one-dimensional (1D) and two-dimensional (2D) SSNMR (Tycko, 2001) techniques to provide structural and dynamic insight into full-length amelogenin as nanospheres and bound to HAP using the properties of ^{13}C chemical shifts, which are

MRGSHHHHHHGSP²LPPHPGSPGYINLSYEVLTPK²⁴WYQSMIRQPYPSYGYEPMGGWLHHQIIPVLSQQHPPSHTLQPHHLLPVVPAQQPVAPQQPMMPVPGHHSMTPTQHHQPNIPPSAQQPFQQPFQQAIP PQSHQPMQPQSPLHPMQPLAPQPPLPPLFSMQPLSPILPELPLEAWPATDK¹⁷³TK¹⁷⁵REEVD¹⁸⁰

Scheme. Full-length sequence of murine amelogenin with the ¹³C, ¹⁵N-labeled lysine residues highlighted in bold (M180-K) and the N-terminal polyhistidine tag sequence highlighted in bold. The sequence is numbered starting with the first native residue, P2.

predictably correlated to protein secondary structure (Wuthrich, 1986). Disordered regions in the protein typically display poor chemical shift dispersion, an observation made for amelogenin monomers in solution (Buchko *et al.*, 2008; Delac *et al.*, 2009; Zhang *et al.*, 2011). This was also observed for both the nanosphere-gel and mineralized samples prepared with uniformly ¹³C-, ¹⁵N-labeled amelogenin (Appendix Fig. 1), precluding extraction of residue assignments and structural data. To simplify the spectra, we chose a minimal labeling scheme which included only the 3 lysine residues (K24, K173, K175), M180-K (see Scheme). Because the charged residues are believed to be involved in the interaction with the HAP surface, and each lysine is strategically located in either the N- or C-terminus of amelogenin, regions thought to be responsible for protein-protein and protein-crystal interactions, these residues are likely to offer insight into the amelogenin-amelogenin and the amelogenin-HAP interface.

Materials & Methods

See the Appendix for additional details.

Nanosphere-gel Preparation

M180-K (30 mL of 20 mM Tris-HCl, pH 8.0, 1.5 mg/mL, 0.07 mM) was stirred for 30 min at room temperature and left undisturbed for 30 min. Ultracentrifugation at 55,000 rpm (259,000 g) for 4 hrs at 20°C resulted in a gel-like coagulate with an estimated protein concentration similar to that found in developing enamel, 300 mg/mL (Robinson *et al.*, 1988). TEM of the nanosphere-gel is shown in Appendix Fig. 2.

Mineralization Experiments

In accordance with published procedures (Fang *et al.*, 2011), lyophilized amelogenin (50 mg) was dissolved in water (1 mg/mL, 0.5 mM), and the pH was adjusted to 3.0 with 0.1 mM HCl to ensure monomeric amelogenin (Buchko *et al.*, 2008). Under slow purging with N₂ gas, aliquots of CaCl₂ and KH₂PO₄ solutions were added until final concentrations of 2.5 mM CaCl₂ and 1.5 mM KH₂PO₄ were reached. The pH was then raised to 7.8, leading to the co-precipitation of amelogenin associated with bundles of needle-like calcium phosphate crystals (Appendix Fig. 3). The solution was incubated at 37°C for 24 hrs before centrifugation at 4,000 rpm for 5 min at 20°C.

Figure 1. The ¹³C 1D-NMR spectra of M180-K in the nanosphere-gel (pH = 8.0, 300 mg/mL, 20 mM Tris-HCl) (a,b) and bound to HAP crystals (c,d) collected at 37°C, with either a DP (a,c) or ¹H-¹³C CP (b,d) NMR experiment. Random coil (+) and β-strand (*) secondary structures are observed, with a significant increase in the β-strand population in the mineralized sample. The vertical lines follow the ¹³C', ¹³C_α, and ¹³C_β set of β-strand resonances.

Solid-state NMR Experiments

All NMR experiments were conducted at a ¹H resonance frequency of 500 MHz in 4 mm rotors with standard pulse programs.

RESULTS

1D-NMR

The ¹³C 1D-NMR spectra of M180-K in the nanosphere-gel form (pH = 8.0, 300 mg/mL, 20 mM Tris-HCl) and the mineralized form are shown in Fig. 1. Two experiments were performed for each sample to obtain both structural and dynamic information. The direct ¹³C 90° pulse (DP) is similar to a solution-state experiment and is not selective to molecules with different mobilities. The ¹H-¹³C cross-polarization experiment (CP) is sensitive to molecular motion, since only relatively rigid nuclei with strong ¹H-¹³C dipolar coupling result in strong CP signals. Both experiments provide structural insight.

Table. Summary of the Solid-state lysine ^{13}C Chemical Shifts for the Spectra in Fig. 1

M180 sample	$^{13}\text{C}'$	$^{13}\text{C}_\alpha$	$^{13}\text{C}_\beta$	$^{13}\text{C}_\gamma$	$^{13}\text{C}_\delta$	$^{13}\text{C}_\epsilon$
Nanosphere						
DP	†175.9	†55.7	†32.1	†23.9	†28.3	†41.5
CP	*173.0 †174.7	*54.2 †56.0	*38.0 †31.3	*25.1 –	*28.2 –	*41.1 –
Mineralized						
DP	*173.7 †176.6	*54.6 †56.0	*38.6 †32.8	*25.7 †25.3	*28.9 †28.5	*41.6 –
CP	*173.7 †176.6	*54.6 †56.0	*38.6 –	*25.7 –	*28.9 –	*41.6 –
Monomer ^a						
K24	176.8	56.6	32.9	24.8	–	–
K173	176.9	57.0	32.5	–	–	–
K175	–	56.3	32.6	24.4	29.4	–
Random coil values (Wishart <i>et al.</i> , 1995)	176.4	56.2	33.1	24.7	29.0	41.9

Estimated errors are ± 0.5 ppm. ^aSolution-state lysine ^{13}C chemical shifts are included for comparison (Buchko *et al.*, 2008). The dagger corresponds to the set of resonances (C' , C_α , C_β) in one protein conformation (assigned as random coil). The asterisk corresponds to resonances of the protein in a second conformation (assigned as beta sheet).

2D-NMR

Two-dimensional ^{13}C - ^{13}C correlation data were collected for the nanosphere-gel and mineralized M180-K samples to aid in the ^{13}C chemical shift assignments. These experiments utilized either ^1H - ^{13}C CP or DP for preparation with dipolar-assisted rotational resonance (DARR) mixing.

DISCUSSION

Amelogenin Nanosphere-gel (1D-NMR)

For the nanosphere-gel sample, the ^{13}C NMR spectrum obtained from the CP experiment (Fig. 1b) generally showed broader lines compared with those of the spectrum from the DP experiment (Fig. 1a) and required 10 times more scans to obtain a spectrum with a similar signal-to-noise ratio. The significant reduction in the signal-to-noise ratio for the CP experiment is consistent with a high mobility at the lysine residues in the nanosphere-gel. The measurement of T_2 relaxation values provided further evidence of fast motion for the nanosphere-gel sample (see Appendix Table). Although nanospheres are large oligomers, the loose packing of the monomers within the nanosphere observed by Cryo-EM (Fang *et al.*, 2011) and the high hydration level are consistent with the high mobility observed here. Our results are also consistent with solution-exposed and flexible N- and/or C- termini in the nanosphere, a structural model that has been previously proposed (Aichmayer *et al.*, 2005).

The 1D-NMR spectrum for the nanosphere-gel sample was also evaluated for structural features. Only one major peak corresponding to each of the 6 lysine carbons ($^{13}\text{C}'$, $^{13}\text{C}_\alpha$, $^{13}\text{C}_\beta$, $^{13}\text{C}_\gamma$, $^{13}\text{C}_\delta$, $^{13}\text{C}_\epsilon$) is observed in the DP- ^{13}C NMR spectrum ($^{13}\text{C}'$, $^{13}\text{C}_\alpha$, and $^{13}\text{C}_\beta$ indicated with a †; Fig. 1, Table), suggesting that all 3 lysines are in similar structural regions. [The resonances at 13, 17, 50, 63, and 179 ppm are likely due to scrambling of the labels during protein expression, based on their lack of correlation in the 2D spectrum.] These chemical shifts deviate little from standard random coil values (Wishart *et al.*, 1995), indicating that

the 3 lysine residues are in random coil regions of the protein (Table). This set of resonances is also observed in the CP spectrum; however, the linewidths are significantly broader relative to the DP spectrum, suggesting a distribution of protein conformations or conformational exchange at an intermediate timescale. While only one set of lysine resonances dominates the DP spectrum, a second set of resonances emerges in the CP spectrum, with assigned lysine $^{13}\text{C}'$, $^{13}\text{C}_\alpha$, and $^{13}\text{C}_\beta$ resonances at 173.0, 54.2, and 38.3 ppm, respectively (*, Fig. 1, Table). For this second set of resonances, the negative deviations of the observed $^{13}\text{C}'$ and $^{13}\text{C}_\alpha$ chemical shifts relative to random coil values ($\Delta\delta^{13}\text{C} = \delta_{\text{Observed}} - \delta_{\text{Random coil}}$), coupled with a positive $\Delta\delta^{13}\text{C}$ for C_β , are representative of a β -strand secondary structure. The detection of a small population of β -strand structure in the nanosphere-gel by SSNMR methods is consistent with previous reports of β -strand structure in amelogenin nanospheres detected by circular dichroism spectroscopy (most recently, Lakshminarayanan *et al.*, 2007) and FT-IR (Beniash *et al.*, 2012). In summary, the analysis of the ^{13}C 1D-NMR spectra of the nanosphere-gel suggests that regions about the lysine residues are dynamic and largely unstructured.

Amelogenin on HAP (1D-NMR)

In comparison with the nanosphere-gel sample, the mineralized sample shows very different structural and dynamic properties. Relative to the DP experiment (Fig. 1c), similar signal-to-noise ratios are observed for the CP experiment (Fig. 1d), and longer R_2 values are obtained than observed for the nanosphere-gel sample (see Appendix Table 1), indicating that molecular motion near the lysine sites of M180-K is reduced in the mineralized sample. The ^{13}C linewidths are similar in both the DP and CP spectra, and relatively narrow in comparison with those of the nanosphere-gel in the CP spectrum (Fig. 1b), suggesting that there is no intermediate timescale exchange and/or limited structural dispersion. However, molecular motion is still evident, since the CP spectrum does not have a significantly

stronger signal than the DP spectrum, the expected enhancement for a rigidly structured sample (Pines *et al.*, 1973). Other evidence of motion is described below. The observation of some protein mobility suggests that the interface between amelogenin and HAP in the lysine regions allows for flexibility, an observation made for regions of both LRAP and statherin bound to HAP (Shaw *et al.*, 2000; Shaw and Ferris, 2008) that may be related to function, such as allowing a single protein to cover more growth sites or serving a second function, such as protein binding.

As observed in the nanosphere-gel sample, 2 distinct sets of ^{13}C resonances are observed in both the DP and CP spectra of the mineralized sample (Figs. 1c, 1d) that also correspond to random coil ($\dagger$) and β -strand ($*$) secondary structure; however, in this case, the β -strand structure is the predominant structural motif. In the CP experiment, the random coil resonances ($\dagger$) are moderately suppressed, suggesting that lysine residues in random coil regions are more mobile than lysine residues in β -strand regions ($*$), which are not suppressed significantly in the CP spectrum. We propose that the more limited mobility for the β -strand lysines compared with the random coil lysines originates from direct association of amelogenin with the mineral. The TEM image (Appendix Fig. 3) shows a low-electron-density protein layer surrounding the HAP crystal, observed previously (Fang *et al.*, 2011). Because this layer is often thicker than the expected monomer thickness (5 nm) (Tarasevich *et al.*, 2009a), we suggest that there is an amelogenin layer adsorbed on top of the amelogenin monomers which are interacting directly with HAP. This amelogenin over-layer was observed previously with AFM studies (Tarasevich *et al.*, 2009a,b). Based on the sample preparation conditions, this protein over-layer is in immediate contact with the amelogenin-HAP complex. We suggest that the higher mobility, random coil lysine component corresponds to this overlying amelogenin multilayer.

An alternative interpretation of the data is that the 2 sets of ^{13}C lysine resonances arise from different lysine environments within the same amelogenin molecule. This would result if part of the amelogenin molecule interacts strongly with the HAP surface (for instance, the C-terminus), while the other part (for instance, the N-terminus) does not. Analysis of previous SSNMR data with LRAP showed that both the N- and C-termini interacted tightly with HAP surfaces, whether phosphorylated at S16 or not, although the N-terminal K24 residue was slightly farther from the surface (7.5 Å to 9.0 Å) and became less structured for the non-phosphorylated sample (Shaw *et al.*, 2004; Shaw and Ferris, 2008; Masica *et al.*, 2011). The recombinant sample reported here is also non-phosphorylated, and it is possible that a looser interaction of the N-terminus could result in a similar increase in mobility and loss of structure. However, we believe that such an interpretation is less likely, given the 2 different amelogenin environments suggested by the thick protein layer observed in the TEM data.

A recent FT-IR study reports that amelogenin has regions of β -sheet structure in the nanosphere form and, upon association with HAP crystals, loses some degree of the well-defined structure (Beniash *et al.*, 2012). This apparent discrepancy may be due to the differences in sample preparation (nanosphere vs. nanosphere-gel), but could also arise from the different information that the 2 techniques provide. While NMR provides precise information at the residue level, FT-IR provides average information

Figure 2. (top) The 2D ^{13}C - ^{13}C correlation spectrum of the M180-K nanosphere-gel, with ^1H - ^{13}C CP for preparation and DARR (dipolar-assisted rotational resonance) mixing at -35°C . (bottom) The 2D ^{13}C - ^{13}C correlation spectra with DARR mixing of M180-K bound to calcium phosphate crystals at 37°C . CP (red), DP (blue). The black (β -strand) and green (random coil) traces are shown to distinguish the 2 structures.

over the entire protein. Our NMR results suggest that the lysines become more structured upon interaction with HAP, while analysis of the FT-IR data indicates that other regions of the protein become less structured. Further NMR evidence is necessary to provide additional residue-specific structural information.

2D-NMR

For the nanosphere-gel sample, the 2D-CP spectrum was obtained at -35°C because of the poor signal intensity at 37°C . As shown in Fig. 2, the spectrum at -35°C was complicated with

significant cross-peak overlap, indicating a distribution of many protein conformations. The 2D-DP ^{13}C - ^{13}C correlation spectrum for the nanosphere-gel sample obtained at 37°C contained no cross-peaks, consistent with the dynamic nature of the lysines in the nanosphere-gel (unpublished observations).

For the mineralized sample, both the 2D-CP and -DP ^{13}C - ^{13}C correlation data were collected at 37°C. As shown in Fig. 2 (bottom), 2 resolved sets of correlated ^{13}C chemical shifts were identified corresponding to lysine residues in random coil (green) and β -strand (black) regions. As observed in the 1D spectra, the lysine peaks corresponding to the random coil chemical shifts (green trace) are suppressed in the 2D-CP spectrum. Our ability to collect interpretable data at 37°C for amelogenin bound to HAP, while it was necessary to go to -35°C to collect (uninterpretable) data for the nanosphere-gel, suggests that amelogenin is significantly more ordered when bound to HAP.

Based on the collective SSNMR and microscopy observations, 2 conclusions can be made. First, in the region of the lysine residues, protein-protein interactions do not stabilize a unique structure, based on the heterogeneous structure and high mobility for the nanosphere-gel sample and the proposed amelogenin over-layer in the mineralized sample. The second conclusion is that a shift to a less dynamic, more homogeneous β -strand structure in the region of the lysines occurs when amelogenin is bound to HAP. The structural changes observed for amelogenin in these 2 environments important for enamel development provide mounting evidence of the importance of structural flexibility for correct amelogenin function.

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