Improved protocol to purify untagged amelogenin - Application to murine amelogenin containing the equivalent P70~~Q~~T point mutation observed in human *amelogenesis imperfecta*.

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ABSTRACT

Amelogenin is the predominant extracellular protein responsible for converting carbonated hydroxyapatite into dental enamel, the hardest and most heavily mineralized tissue in vertebrates. Despite much effort, the precise mechanism by which amelogenin regulates enamel formation is not fully understood. To assist efforts aimed at understanding the biochemical mechanism of enamel formation, more facile protocols to purify recombinantly expressed amelogenin, ideally without any tag to assist affinity purification, are advantageous. Here we describe an improved method to purify milligram quantities of amelogenin that exploits its high solubility in 2% glacial acetic acid under conditions of low ionic strength. The method involves heating the frozen cell pellet for two 15 min periods at ~70 °C with two minutes of sonication in between, dialysis twice in 2% acetic acid (1:250 v/v), and reverse phase chromatography. A further improvement in yield is obtained by resuspending the frozen cell pellet in 6 M guanidine hydrochloride in the first step. The acetic acid heating method is illustrated with a murine amelogenin containing the corresponding P70~~Q~~T point mutation observed in an human amelogenin associated with *amelogenesis imperfecta* (P71T), while the guanidine hydrochloride heating method is illustrated with wild type murine amelogenin (M180). The self-assembly properties of P71T were probed by NMR chemical shift perturbation studies as a function of protein (0.1 to 1.8 mM) and NaCl (0 to 367 mM) concentration. Relative to similar studies with wild type murine amelogenin, P71T self-associates at lower protein or salt concentrations with the interactions initiated near the N-terminus.

Keywords: *amelogenesis imperfecta*; intrinsic disorder; amelogenin; tooth enamel; biomineralization; NMR spectroscopy.

Abbreviations used: hM180, full-length murine amelogenin with a 12-residue N-terminal tag; hP41T, murine amelogenin with a P41T point mutation and 12-residue N-terminal tag; P71T, murine amelogenin with a P71T point mutation; hT21I, murine amelogenin with a T21I point mutation and 12-residue N-terminal tag; M180, full-length murine amelogenin; NMR, nuclear magnetic resonance; OD, optical density.

Introduction

In the final stages of tooth development a 1-2 mm layer of enamel is deposited over the dentin [1]. Once mineralization is complete the enamel is not remodeled or repaired. Consequently, enamel has to last a lifetime, withstanding abrasion and fracture during mastication within the bacteria-filled environment of the mouth. Wear-resistance is achieved with a unique combination of high mineral content and high degree of architectural organization [2] that makes enamel the hardest tissue in the vertebrate body [1,3]. By weight, over 97% of mature enamel consists of carbonated hydroxyapatite crystals elongated in the c-axial direction and tightly packed into parallel arrays intricately crossed weaved into a unique lattice [4-6]. This process, amelogenesis, occurs over three stages (secretory, transition, and maturation) [7] with the cascade of events beginning with dentin triggered differentiation of specialized cells into ameloblasts. In the secretory-stage these ameloblasts secrete a cocktail of proteins into the extracellular environment around the dentin with enamel nucleation and growth occurring almost immediately thereafter [6,8-11]. During the transition-stage there is a progressive removal of matrix proteins by proteases, such as enamelysin (MMP-20) and kallikrein (KLK-4). At the end of the maturation-stage hard and tough enamel, largely devoid of organic material, remains to protect the dentin and other internal parts of the tooth for life.

Amelogenin is the primary protein secreted by ameloblasts and is largely responsible for guiding the nucleation, growth, and organization of brittle hydroxyapatite into tough enamel [6,8,11-13]. This low-molecular-weight protein (~ 22 kDa) is highly conserved among species [14] and can be divided into three parts as illustrated for murine amelogenin in Figure 1: an N-terminal tyrosine-rich region (TRAP) containing a post-translationally phosphorylated serine (S16), a central region rich in P, L, H, and Q residues (HQP-rich region), and a hydrophilic C-

terminal region. The charged amino acid residues at both termini are highly conserved suggesting that these regions serve important functional roles for mediating enamel biomineralization [15]. The central region is exclusively hydrophobic and responsible for one of the unique properties of amelogenin, an ability, under the proper environmental conditions present in the secretory-stage, to self-assemble into a quaternary structure, 40 - 100 nm in diameter, called a nanosphere [10]. These nanosphere structures have been observed both *in vivo* [10] and *in vitro* [9] and are believed to be an important functional form of the protein [16]. Assembly of individual amelogenin molecules into larger units is hypothesized to occur progressively [17,18] with the process dependent on the sensitive interplay between protein concentration and the solution properties (ionic strength, pH, solutes, and temperature) [18-22].

Amelogenesis imperfecta [23] is a group of hereditary conditions associated with six genes that affect the quantity and quality of enamel [24,25]. Clinical phenotypes of the mutations to these genes vary and include hypomaturation, hypoplasia, and hypocalcification. The majority of genetic mutations associated with *amelogenesis imperfecta* is in the gene for amelogenin [26] and includes a single amino acid substitution, P70~~Q~~T, in the primary amino acid sequence of human amelogenin [27]. The phenotype of the P70~~Q~~T mutation is hypomineralized enamel with a higher than normal protein content [27]. *In vitro* studies showed that proteolysis by MMP20 was decelerated in amelogenin containing the P70~~Q~~T substitution [28] and relative to wild type amelogenin, this mutated form of the protein formed larger nanospheres [29]. Two other single point mutations identified in the amelogenin gene, T21~~Q~~I and P40~~Q~~T, were also shown to affect the self-assembly properties of amelogenin relative to the wild type protein [26,30,31].

The ability to express and purify large quantities of amelogenin is important for studies aimed at understanding its role in biomineralization. This is especially important for solution- and solid-state NMR experiments that require upwards of 5 and 50 milligrams of protein, respectively, for a single sample. In addition, amelogenin and its splice variants/proteolytic digestion products may have biological properties similar to signaling molecules [32,33], and consequently, potential clinical uses [34]. Amelogenin also has a proposed role in the synthesis of novel biomaterials [35] and as a fusion partner to purify target proteins/peptides [36]. The later potential use for amelogenin exploits the protein's high solubility under acidic conditions (2% acetic acid) [37] and enables the purification of untagged recombinant amelogenin in a one-step protocol [36]. The reported one-step protocol involves heat treating cells (80 °C) in 3% acetic acid and isolating the soluble fraction by centrifugation to yield amelogenin that is ~95% pure. While yields of up to 1 g/L were reported using TB-medium, we could not duplicate these yields using minimal media necessary to incorporate NMR isotopes (nitrogen-15 and carbon-13). Here, we report modifications that increase the yield in minimal media and allow the facile preparation of large quantities of carbon-13 and/or nitrogen-15 labeled amelogenin necessary for solution- and solid-state NMR spectroscopy. Our modifications to the purification protocol are demonstrated with untagged murine amelogenin (M180) and an untagged murine amelogenin containing the corresponding P70 Δ I point mutation observed in human amelogenin that is associated with *amelogenesis imperfecta* (P71 Δ I, P71T, relative to human amelogenin, murine amelogenin contains an extra methionine at the 29th position). The ^1H - ^{15}N HSQC NMR spectrum of M180 in 2% acetic acid is compared to the previously reported spectrum for M180 containing a 12-residue, hexa-histidine tag (MRGSHHHHHHGS-, hM180). To probe the finer molecular details of how the P71 Δ I point mutation may affect the self-assembly properties of P71T, this

protein was characterized in solution using NMR spectroscopy under conditions that promote self-assembly, increasing protein concentration and ionic strength [18,22,38].

Materials and methods

All chemicals were purchased from Research Products International Corporation (Mount Prospect, IL) except trifluoroacetic acid (TFA), glacial acetic acid, and HPLC grade acetonitrile (Sigma Chemical Company, St. Louis, MI).

Construction of expression vectors and protein expression

The genes for full-length wild type murine amelogenin (M180) [39] plus the site directed P71 ~~Q~~T mutation (P71T), codon-optimized for *Escherichia coli* expression, were synthesized and inserted into the expression vector pJexpress414 (DNA 2.0 Inc, Menlo Park, CA). The recombinant plasmids were transformed into *E. coli* BL21(DE3) cells (Novagen, Madison, WI) following a heat shock method. Using the antibiotic ampicillin (150 μ g/mL), 15% glycerol stocks (1 mL, OD₆₀₀ $\approx$ 0.8) were prepared (from single colonies grown on LB agar plates) and stored at -80 °C until ready for use. Uniformly ¹⁵N-labeled protein was prepared using an autoinduction protocol [40] starting with a 20 mL LB culture (37 °C) seeded with 1 mL of frozen glycerol stock. At an OD₆₀₀ $\approx$ 0.8, the 20 mL culture was directly transferred to 500 mL of autoinduction minimal media. Growth continued at 37 °C until an OD₆₀₀ of $\sim$ 0.8 was reached

and the cell culture transferred to a 25 °C shaker. Following overnight incubation the cells were harvested by mild centrifugation and stored at -80 °C. Uniformly ^{15}N -, ^{13}C -labelled P71T and M180 were obtained in a similar manner except for the transfer of the 20 mL LB culture into 750 mL of minimal media (Miller) containing $^{15}\text{NH}_4\text{Cl}$ (1 mg/mL) and D- $^{13}\text{C}_6$ glucose (2.0 mg/mL), NaCl (50 $\mu\text{g/mL}$), MgSO_4 (120 $\mu\text{g/mL}$), CaCl_2 (11 $\mu\text{g/mL}$), and ampicillin (150 $\mu\text{g/mL}$). When the cell culture reached an OD_{600} reading of ~ 0.8 , it was transferred to a 298 K incubator and protein expression induced with isopropyl β -D-1-thiogalactopyranoside (0.026 $\mu\text{g/mL}$). After four hours the cells were harvested by mild centrifugation and stored at -80 °C.

Protein purification

The frozen pellet was resuspended (500 mL autoinduction minimal media (^{15}N -labeled) or 750 mL M9 minimal media (^{13}C - and ^{15}N -labeled)) in 40 mL of 2% glacial acetic acid and placed in a 70 °C water bath for 15 minutes. The suspension was then sonicated (without cooling) for 2 minutes and returned to the 70 °C for an additional 15 minutes. To demonstrate the advantage of dialysis, the lysed cells from a 500 mL autoinduction growth was split into two equal fractions and resuspended in 20 mL of 2% acetic acid. For one fraction, the insoluble cell debris was immediately removed by centrifugation at 25,000g for 1 h. For the other fraction, the solution was placed in dialysis tubing with a 3,500 dalton molecular weight cutoff (Fisher Scientific, Waltham, MA) and dialyzed sequentially against five liters of 1% (~ 6 h) and 2% (~ 12 h) glacial acetic acid (1:250, v:v) before removing the insoluble cell debris in a similar centrifugation manner. The supernatants were applied onto an XBridge Prep C18 (5 μm , 10x250 mm) reverse phase column (Waters, Milford, MA) with the following purification gradient

applied after loading the material using 100% Buffer A (Buffer A = water in 0.1% TFA, Buffer B = 70% aq. CH₃CN in 0.1% TFA, flow rate 2.5 mL/min: Step 1 - 0.5 CV, 100% Buffer A; Step 2 - 0.2 CV, linear gradient 0 to 40% Buffer B; Step 3 - 4.0 CV, linear gradient 40 to 100% Buffer B). The fractions containing amelogenin eluted at ~ 68% Buffer B. These fractions were pooled, frozen at -80 °C, and lyophilized. After weighing on an analytical scale, the material was resuspended in the appropriate buffer for subsequent analyses (an NMR buffer consisting of 2% CD₃CO₂D, 7% D₂O/91% H₂O, pH 2.8) [22].

Further improvement in the amelogenin yields were obtained using the above protocol except for the resuspension of the frozen pellet (500 mL autoinduction minimal media (¹⁵N-labeled) or 750 mL M9 minimal media (¹³C- and ¹⁵N-labeled)) in 40 mL of 6 M guanidine hydrochloride (unadjusted pH ~4.8). To demonstrate the advantage of using the chaotropic agent, harvested cells from a 500 mL autoinduction growth of M180 was split into two equal fractions with one fraction resuspended in 20 mL of 6 M guanidine hydrochloride solution and the other in 20 mL of 2% glacial acetic acid. Both solutions were heat treated as described above with the 2% acetic acid solution processed in an identical manner. On the other hand, the 6 M guanidine hydrochloride solution was first centrifuged for 25,000g for 1 h to remove cellular debris and the supernatant then dialyzed twice in 5 L of 2% glacial acetic acid. Following dialysis, precipitated material was removed by another centrifugation at 25,000g for 45 min with the products in the supernatant analyzed by reverse phase HPLC chromatography and SDS PAGE.

NMR analysis

All NMR data was collected at 20 °C using a Varian Inova-750 spectrometer and Biopack pulse programs. Three-dimensional HNCACB data was collected on the double-labeled M180 and P71T samples to verify the amide chemical shift assignments that were otherwise based primarily on comparison to the ^1H - ^{15}N HSQC spectrum of previously assigned hM180 containing the non-removable N-terminal tag MRGSHHHHHHGS- [37].

For the dilution study, an initial 1.8 mM sample of P71T was prepared in the NMR buffer. The initial ^1H - ^{15}N HSQC was collected on a 250 μL sample in a Shigemi NMR tube (Shigemi Inc., Allison Park, PA) with subsequent spectra collected following serial dilutions to 0.9, 0.45, 0.22, and 0.1 mM. For the NaCl titration study, a 2M NaCl stock solution was prepared in NMR buffer. Seven and one half microliter aliquots of the sodium chloride stock solution were added directly to the amelogenin sample (0.25 mM (300 μL)) and, following gentle agitation, a high resolution ^1H - ^{15}N HSQC spectrum (20 °C) was immediately acquired. NMR spectra were recorded in the absence of salt and at NaCl:protein molar ratios of 200, 400, 600, 800, 1000, 1200, 1400, 1600, and 1800 to one. At the end of the titration, due to dilution, the final concentration of P71T and NaCl was 0.204 mM and 367 mM, respectively, in a final volume of 368 μL . All NMR data was processed using Felix2007 (Felix NMR, Inc, San Diego, CA) and analyzed using Sparky (v 3.115) [41]. The ^1H , ^{13}C , and ^{15}N chemical shifts referenced to DSS (DSS = 0 ppm) using indirect methods [42].

Results

Protein purification.

Recombinant human, porcine, and marine amelogenin have been used in many studies with the general purification protocol following the method first reported by Simmer *et al.* [43]: cell lysis under denaturing conditions (6 M guanidine hydrochloride), centrifugation, ammonium sulfate precipitation, and reverse phase chromatography. The yields of amelogenin reported using this method are modest (4 -11 mg/L cell culture) [36] and may be improved (up to 70 mg/L) by adding an N-terminal histidine tag to the protein [22, 31, 44]. Unfortunately, the nanosphere forming properties of amelogenin in solutions of high ionic strength or pH values above 6, conditions employed by common proteases, makes the removal of many terminal polyhistidine tags difficult. Hence, the one-step protocol reported by Bonde *et al.* [36] without a purification tag is an attractive alternative to prepare large quantities (10s of mg) of amelogenin. Our initial efforts to prepare ¹⁵N-labeled hM180 following the one-step protocol using autoinduction minimal media produced much poorer yields than our standard method using metal-affinity chromatography [31]. These yields were reduced further when using an M9 minimal media protocol with isopropyl β -D-1-thiogalactopyranoside (IPTG) induction to generate ¹³C- and ¹⁵N-labeled protein (data not shown). Because Bonde *et al.* [36] did report that their yields increased when the volume of acetic acid used for cell lysis was increased from 60 to 600 mL (for a 1 liter culture), we surmised that the high ionic strength of the cell lysis solution in 3% acetic acid might result in amelogenin aggregation [22] and much of its removal in the cell pellet following centrifugation. Therefore, instead of lysing the cells in a large volume of acetic acid solution to lower the ionic strength we decided to reduce the ionic strength by dialyzing the cell lysis fraction in an acetic acid solution.

The P71T cells from a 500 mL autoinduction growth in minimal media containing $^{15}\text{NHCl}_4$ were divided equally and resuspended in 20 mL of 2% acetic acid. Both fractions were lysed similarly following a combination protocol described by Bonde *et al.* [36]: 15 min in a 70 °C water bath, 2 min sonication while warm, and a further 15 min in a 70 °C water bath. The cell debris for one fraction was removed by centrifugation and the supernatant assayed by SDS PAGE and reverse phase HPLC. For the other fraction the entire cell lysis material was placed in dialysis tubing with a 3,500 MW cut-off and dialyzed sequentially in 5 L of 1% acetic acid followed by 5 L of 2% acetic acid (5 °C) before removing the cell debris by centrifugation and analyzing the supernatant by SDS-PAGE and reverse phase HPLC. The SDS-PAGE gel shown in Figure 2 illustrates the expression of a dominant band in the total cell lysis fraction (Lane A) with a molecular weight of ~22 kDa, as expected for P71T. Upon centrifugation, the major product in the supernatant is still the band corresponding to P71T with many of the impurities removed (Lane 0). The intensity of this dominant band increases over two 1:250 (v:v) dialyses (Lanes 1 and 2). A better quantification of the products and impurities in the various fractions is afforded by reverse phase HPLC as illustrated in Figure 3A, profiles of the supernatant following centrifugation of the fraction without (red) and following 2x dialysis (black). Clearly, there is an ~ 3-fold increase in the band at ~36 min corresponding to P71T. Moreover, following dialysis there is a substantial decrease in the band corresponding to unknown impurities that elutes at ~ 23 min and a yellow/brown tinge color of the supernatant without dialysis is eliminated following two dialysis cycles. In summary, dialysis of the cell lysis material in 2% acetic before centrifugation increases the yield of amelogenin and reduces the impurities in the supernatant. Further purification by reverse phase chromatography removes impurities present after dialysis.

For P71T using a minimal media autoinduction protocol the yield of ^{15}N -labeled amelogenin improved from ~50 mg/L to almost 150 mg/L.

While the SDS PAGE in Figure 2 shows that the amount of amelogenin in the soluble fraction increases with dialysis in 2% acetic acid, the intensity of the P71T band in Lane A was greater than the corresponding band in Lane 2 suggesting that much amelogenin is lost in the cell pellet during centrifugation. To determine if this amelogenin could be recovered and the yield further improved, the cell pellet following centrifugation after 2x dialysis in acetic acid was resolubilized in 20 mL of 6 M guanidine hydrochloride and then dialyzed 2x in 5 L of 2% acetic acid. During dialysis, a substantial amount of material precipitated out of solution. This precipitant was removed by centrifugation and the soluble material present in this fraction is illustrated in Lane G in Figure 2. Clearly, the 6 M guanidine hydrochloride treatment recovered more P71T. While additional impurities are present in this fraction, they are eliminated by reverse phase HPLC chromatography (Figure 2, Lane 4).

To determine if better yields of amelogenin could be obtained by heating in 6 M guanidine hydrochloride instead of 2% acetic acid, M180 cells from a 500 mL autoinduction growth in minimal media containing $^{15}\text{NHCl}_4$ were divided equally with one fraction resuspended in 2% acetic acid (20 mL) and the other fraction resuspended in 6 M guanidine hydrochloride (20 mL). Both fractions were lysed as described for P71T. Insoluble cell debris from the 6 M guanidine hydrochloride fraction was removed by centrifugation prior to dialysis of the supernatant 2x in 5 L of 2% acetic acid. Again, during dialysis a substantial amount of material precipitated out of solution. Figure 3B is the reverse phase HPLC profile of the M180 supernatant following centrifugation for the fraction lysed in 2% acetic acid (black) and guanidine hydrochloride (purple). While there are a few additional minor impurities from the

fraction lysed in guanidine hydrochloride, there is an ~ 2-fold increase in the band at ~ 36 min corresponding to M180. In summary, the yield of amelogenin can be further improved by heat lysing the cells in 6 M guanidinium hydrochloride instead of 2% acetic acid prior to dialysis in 2% acetic acid. While this protocol results in some additional impurities in the supernatant following dialysis, most can be removed by reverse phase chromatography. For M180 using a minimum media autoinduction protocol the yield of ^{15}N -labeled amelogenin improved from ~120 mg/L to over 200 mg/L.

Note that while SDS PAGE analysis suggested the supernatant after dialysis and centrifugation was quite pure for P71T cells lysed in 2% acetic acid, the supernatant did have a faint yellow/brown color. This color was more pronounced for the cells lysed in 6 M guanidine hydrochloride. Using either method, this color disappeared following reverse phase HPLC chromatography suggesting that the second purification step is beneficial especially since the nature of this impurity is unknown. Regardless of the identity of the impurity, ^1H - ^{15}N HSQC spectra of supernatant with and without dialysis (lyophilization and resuspension in 2% acetic acid at approximately equal protein concentrations) suggested it promoted self-association of the amelogenin. The cross peaks in the spectrum for the undialyzed protein were very broad and many expected resonances were missing while the spectrum following dialysis was much sharper and complete (*vida infra*, data not shown). Hence, for the NMR studies for P71T and M180 described below, both proteins were further purified by reverse phase chromatography after heat treatment and dialysis.

Structural effects of the N-terminal tag and the P71~~Q~~T point mutation

Figure 4 is an overlay of the ^1H - ^{15}N HSQC spectrum of M180 and hM180 under conditions where hM180 was previously shown to be monomeric in solution (0.3 mM protein concentration, 2% acetic acid, pH 2.8, 20 °C) [22]. The most noticeable differences are the additional resonances due to the N-terminal tag in hM180 (labeled in red and an asterisk) and the amide chemical shifts for L3 (labeled and circled blue). The latter observation is most likely because L3 is the penultimate residue at the N-terminus of M180 (the N-terminal methionine is removed in *E. coli* by methionine aminopeptidase) [36] but is many residues removed from the N-terminal residue in hM180. While the other parts of the spectra do not overlap exactly, they are very similar (the proton chemical shift range in Figure 4 is only 0.8 ppm) indicating the N-terminal tag in hM180 has little influence on the structure adopted by the rest of the protein under these conditions, and concomitantly, perhaps little influence on the self-assembly properties of amelogenin under these conditions [22]. Another consequence of the similarity of the ^1H - ^{15}N HSQC spectrum of M180 with hM180 is that a rapid assignment of the backbone $^1\text{H}^{\text{N}}$ and ^{15}N amide resonances for M180 was possible from the previous assignments of hM180 [37] with confirmation with a HNCACB data set for M180.

Figure 5 is an overlay of the ^1H - ^{15}N HSQC spectrum of P71T (yellow) with M180 (black) under conditions where hM180 was previously shown to be monomeric in solution (0.1 mM protein concentration, 2% acetic acid, pH 3, 20 °C) [22]. While the majority of the amide assignments for P71T could be made by comparison to the amide assignments for M180, an HNCACB data set was collected to assign T71 and any residues with significant chemical shift perturbations. The new amide resonance, T71, overlaps with the amide resonance for H91 as illustrated in Figure 5. Aside from three resonances around the site of the P71~~Q~~T substitution

(L70, V72, and V73 circled in Figure 5), the spectra for P71T and M180 overlay well suggesting that the amino acid substitution has little effect on the global structure of amelogenin. Such a highly localized chemical shift effect as a result of a single amino acid substitution is consistent with an intrinsically disordered protein, as reported for porcine [45] and murine [22] amelogenin. Local electronic differences due to the P71~~N~~T change in P71T is likely responsible for the observed chemical shift perturbations. Note that one consequence of assigning the ^1H - ^{15}N HSQC spectra of M180 and P71T was the identification of a mis-assignment in the originally published spectrum for hM180 [37] that exchanges the assignments for V72 and V53.

Protein concentration effects on the self-assembly of P71T

The collection of ^1H - ^{15}N HSQC spectra at different protein concentrations is routinely used to assess concentration dependent self-association or transient aggregation of NMR samples. Here, it was clearly observed that the ^1H - ^{15}N HSQC spectra of P71T changed as the protein concentration was increased from 0.1 mM to 1.8 mM: the amide cross peaks became broader and ten additional peaks disappeared (spectra not shown). These observations suggest that P71T self-associates to form a larger molecular weight complex as the protein concentration is increased [31]. The disappearance of amide cross peaks in the ^1H - ^{15}N HSQC spectrum at higher protein concentrations is likely due to restricted motion and/or heterogeneous interactions at a protein-protein interface as interpreted previously for hM180 as a function of increasing NaCl and CaCl_2 concentrations [22,46]. It was possible to track the disappearance of most of the amide cross peaks in the ^1H - ^{15}N HSQC spectra over the concentration range and this data for

P71T is summarized in Fig. 6 along with previously presented data for two other murine amelogenin mutant proteins associated with *amelogenesis imperfecta*, T21~~Y~~I (hT21I) and P40~~Y~~T (hP41T) [31]. At 1.8 mM concentration, the amide resonances that disappear in the ^1H - ^{15}N HSQC spectrum of P71T are all localized near the N-terminus between V19 – Q32, a region similar to the one containing vanishing resonances in hP41T (T21 – Y37) at this protein concentration. In contrast, hT21I was much more sensitive to concentration and at 1.8 mM, resonances were largely absent for a large N-terminal region between L3 – H58 and a C-terminal region between L142 – A170. Note that for native hM180, no amide resonances disappeared over this concentration range and only a broadening of cross peaks were observed as the protein concentration was increased to 1.8 mM. Overall, these results suggest that the mutated proteins begin to self-assemble at a lower protein concentration than the wild type protein in the order hT21I < hP41T = P71T < hM180.

Sodium chloride effects on self-assembly

By monitoring the disappearance of amide cross peaks in the ^1H - ^{15}N HSQC of hM180, hT21I, and hP41T as a function of increasing NaCl it was previously shown that increasing ionic strength affected a stepwise self-assembly of amelogenin into larger complexes [22, 31]. Like the concentration titrations studies, this association was largely followed by the disappearance of amide resonances in ^1H - ^{15}N HSQC spectra as a function of increasing NaCl concentrations and is summarized for P71T in Figure 7. Furthermore, by collecting a series of ^1H - ^{15}N HSQC spectra at more closely spaced titration points it was possible to more finely identify the regions of P71T

responsible for initiating self-assembly. At the first titration point, a NaCl:P71T molar ratio of 200:1, there was little change in the ^1H - ^{15}N HSQC spectrum of P71T aside from some mild line broadening (hence, this data is not shown in Figure 7). At the next titration point, a NaCl:P71T molar ratio of 400:1, cross peaks for four out of the seven resonances between Y26 and Q32 disappear while the intensity of the remaining resonances change little, suggesting that the self-assembly process is initiated in this region. Upon further increase of the salt concentration additional resonances disappear in a fan-like fashion about this region with all the amide cross peaks for the residues between H6 – L54 gone at a NaCl:P71T molar ratio 1400:1. Near the C-terminal, the first amide resonance to disappear is S152 at a NaCl:P71T molar ratio of 600:1 and at 1400:1 ratio this region has expanded to include the disappearance of all the resonances between L147 – W168. At the final titration point, a NaCl:P71T molar ratio of 1800:1, any resonance that has not disappeared completely is greatly reduced in intensity. In general, a similar pattern was observed for hM180, hT21I, and hP41T as a function of increasing NaCl concentration [31] with self-assembly due to increased NaCl accelerated for hT21I > hP41T > M180. Relative to these later three proteins, P71T behaved most closely to hP41T, as observed in the concentration titration.

Discussion

Using our improved purification approach it is possible to obtain large quantities of isotopically labeled recombinant amelogenin without the need for the introduction of a tag onto the primary sequence to assist protein purification or the use of large volumes of solvent to lyse

the cells. While the NMR data presented here show that the structure for full-length native murine amelogenin, with and without the N-terminal polyhistidine tag, is essentially the same at 0.3 mM protein concentrations in 2% acetic acid, removing the tag removes any concerns about it possibly influencing the self-assembly process, especially under more physiologically relevant conditions. Its removal may be especially important given that the N-terminal region of hM180 is the region first affected in the process of self-assembly induced by increasing salt concentrations [22], a region identified in playing a role in porcine amelogenin self-assembly induced by 2,2,2-trifluoroethanol [47], and a region implicated in self-assembly at a more physiological pH (5.5) by NMR experiments using a series of truncated murine amelogenin sequences [48]. Furthermore, the highly conserved [14] N-terminal region, M1-M42, has been identified by yeast two-hybrid assays [49] as essential for nanosphere self-assembly and it has been postulated that upon nanosphere formation this terminal is surface exposed [9,16,20,49], increasing the nanosphere's solubility and enhancing its interactions with calcium phosphate [15].

At 0.1 mM protein concentration in 2% acetic acid, P71T is a monomeric, intrinsically disordered protein and relative to wild type amelogenin the single P71 ~~Q~~T substitution had little, if any, effect on the protein's structure. As the protein concentration or NaCl concentration was increased, the broadening and eventual disappearance of amide cross peaks in the ^1H - ^{15}N HSQC spectra of P71T suggested the protein was self-associating [22]. As a function of increasing protein concentration, the region affected was V19 – Q32 and as a function of increasing NaCl concentration, the region first affected was Y26 - Q32. Both these regions overlap with K24 - S28, a structural flexible region identified in solid-state NMR studies of the 59-residue splice-

variant amelogenin LRAP [50, 51]. Collectively, these studies suggest this region near the N-terminal of amelogenins may be functionally important.

Hydrophobic interactions are an important force driving the proper folding of proteins into their native structure (intramolecularly) and into their associated/aggregated states (intermolecularly) [52]. Consequently, a simple explanation to explain the increased tendency for P71T to self-associate at lower protein and NaCl concentrations than wild type M180 may be the increase in hydrophobicity due to the replacement of P71 with a more hydrophobic amino acid residue, T. Such an explanation was proposed to explain similar observations with hT21I and hP41T [38], where a P was also replaced with a T (hP41T) and a T was replaced with an I (hT21I). Using the hydrophobicity index of Kyte and Doolittle [53], the T↔I substitution represents a greater increase in hydrophobicity than the P↔I substitution, 5.2 versus 0.9 units. The approximately five-fold difference in hydrophobicity increase in hT21I over hP41T and P71T may be primarily responsible for the stronger tendencies of hT21I to self-associate than hP41T and P71T and the same hydrophobicity increase for P71T and h41 may explain their similar self-association properties. Whatever the physical reason, the self-association of the mutants P71T, hT21I, and hP41T at lower protein concentrations and lower ionic strengths than the tagged wild type hM180 may account for the defective enamel associated with *amelogenesis imperfecta*.

There are a number of ways an increased tendency to self-associate could impair amelogenesis. Premature self-association within ameloblast cells may hinder amelogenin secretion, a mechanism recently reported for the Y34↔H amelogenin mutation associated with *amelogenesis imperfecta* [54]. More tightly associated or large nanospheres may interfere with protein-mineral interactions and proteolytic digestion [26, 30]. Indeed, larger nanospheres that

are not digested as quickly by MMP20 [28] have been reported for the P70~~Q~~^T human amelogenin mutation [29]. Disassembly of amelogenin nanospheres may be necessary for proper enamel formation [55, 56] and a more tightly associated nanosphere may hinder this disassociation.

In summary, by taking advantage of amelogenin's high solubility in 2% glacial acetic acid under conditions of low ionic strength it is possible to prepare milligram quantities of untagged, isotopically labeled, recombinant amelogenin. The highest yields are obtained by solubilizing the cell pellet in 6 M guanidine hydrochloride instead of 2% acetic acid. While relatively pure protein is obtained after extensive dialysis in 2% acetic acid, because nanosphere formation is sensitive to the interplay of protein concentration and the properties of the solution (ionic strength, pH, solutes, and temperature) [18-22], a final purification step using reverse phase chromatography is recommended. Maximizing the yield of recombinant amelogenin is especially important for preparing isotopically labeled protein for solid-state NMR experiments that can require upwards of 50 milligrams of sample [57]. This improvement in yield afforded by these improved protocols will not only facilitate the purification of peptides and proteins that use amelogenin as a fusion partner [36], but also enable the preparation of large quantities of untagged and isotopically labeled amelogenin for structural and biochemical studies of biomineralization that may lead to the elucidation of the molecular mechanism of enamel formation.

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Figure legends

Fig. 1. Primary amino acid sequence of murine amelogenin highlighting the three major regions of the protein: N-terminal tyrosine-rich region, TRAP (cyan); hydrophobic central region rich in P, L, H, and Q residues, HQP-rich region (grey); and a hydrophilic C-terminal region (magenta). The acidic residues are colored red, basic residues colored blue, and the lone serine that is phosphorylated *in vivo* is colored yellow. The location of amino acid substitutions in three point mutations associated with *Amelogenesis imperfecta*, T21~~Q~~I (hT21I), P41~~Q~~T (hP41T), and P71~~Q~~T (P71T), are indicated by a blue box.

Fig. 2. A 8-16% Tris-HCl SDS-PAGE gel illustrating the purity of selected fractions during stages in the purification protocol for P71T. Lane A – After heating and pre-dialysis; Lane 0 – Supernatant of A after centrifugation (0x dialysis); Lane 1 – Supernatant following first 1:250 (v:v) dialysis in 1% acetic acid (1x dialysis); Lane 2 – Supernatant follow second first 1:250 (v:v) dialysis in 2% acetic acid (2x dialysis); Lane G - 6 M guanidine hydrochloride recovery of precipitants from Lane 2 fraction dialyzed 2x in 2% acetic acid (1:250, v:v); Lane 3 – Reverse phase HPLC purification of Lane 2 supernatant; Lane 4 – Reverse phase HPLC purification of Lane G supernatant; M – molecular weight markers.

Fig. 3. Reverse-phase HPLC chromatogram profiles for P71T (A) and M180 (B) illustrating the improvement in yields due to dialysis in 2% acetic acid and cell lysis with 6M guanidine hydrochloride, respectively. In both chromatograms the major band eluting at ~36 min contains

the amelogenin protein and corresponds to ~68% Buffer B (70% aq. CH₃CN in 0.1%). (A) Soluble fraction of the cell lysis material from P71T, prepared by heating in 2% acetic acid, following centrifugation without (red) and with (black) extensive dialysis in 1 – 2 % acetic acid. (B) Soluble fraction of the cell lysis material from M180, prepared by heating in equal volumes of 2% acetic acid (black) or 6M guanidine hydrochloride (purple).

Fig. 4. (A) Overlay of the ¹H-¹⁵N HSQC spectrum of M180 (black) and hM180 (red) collected under similar experimental conditions (0.3 mM protein concentration, 2% acetic acid, pH 2.8, 20 °C) at a ¹H resonance frequency of 750 MHz. The resonances for the 12-residue, N-terminal poly-histidine tag in hM180 are labeled in red and with an asterisk. Aside from the N-terminal tag, the most significant difference between the spectra of the two proteins is the resonance for L3 circled in blue.

Fig. 5. Overlay of the ¹H-¹⁵N HSQC spectra of M180 (black) and P71T (yellow) collected under similar experimental conditions (0.3 mM protein concentration, 2% acetic acid, pH 2.8, 20 °C) at a ¹H resonance frequency of 750 MHz. The amide resonances with the most significant chemical shift differences are circled in blue and labeled. The T71 resonance in P71T, indicated with a blue arrow, overlaps with the H91 amide resonance.

Fig. 6. Summary of the amide resonances that partially disappear (grey-filled circles) or completely disappear (open circles) in the ¹H-¹⁵N HSQC spectra of ¹⁵N-labelled hP41T, hT21I, and P71T when the concentration of the protein is increased from 0.1 to 1.8 mM. Amide cross

peaks whose intensity change little over the concentration range are indicated by solid circles and cross peaks that could not be tracked unambiguously are indicated by green-filled circles. The full murine amelogenin sequence is shown with the proline residues highlighted in cyan and the site of the individual point mutations in each protein highlighted in red. Underneath the primary sequence is a schematic illustration of the three major regions of the protein: TRAP region (cyan); HQP-rich region (grey); and hydrophilic C-terminal region (magenta). The data for hP41T and hT21I were published previously [31].

Fig. 7. Summary of the amide resonances that partially disappear (grey-filled circles) or completely disappear (open circles) in the ^1H - ^{15}N HSQC spectra of ^{15}N -labelled P71T as a function of increasing NaCl concentration. Amide cross peaks whose intensity change little over the concentration range are indicated by solid circles and cross peaks that could not be tracked unambiguously are indicated by green-filled circles. The NaCl:P71T molar ratio are shown on the left. The full murine amelogenin sequence is shown with the proline residues colored cyan and the site of the P71 ~~Q~~T point mutation highlighted in red. Underneath the primary sequence is a schematic illustration of the three major regions of the protein: TRAP region (cyan); HQP-rich region (grey); and hydrophilic C-terminal region (magenta).

Figure 1

MPLPPHPGSP	GYINLSYEVL	TPLKQWYQSMI	RQPYPSYGYE	PMGGWLHHQI
IPVLSQQHPP	SHTLQPHHHL	PVVPAQQPVA	PQQPMPVPG	HHSMTPTQHH
QPNIPPSAQQ	PFQQPFQPQA	IPPQSHQPMQ	PQSPLHPMQP	LAPQPPLPPL
FSMQPLSPIL	PELPLEAWPA	TDKTKREEVD		

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7