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Quantitatively and Kinetically Identifying Binding Motifs of Amelogenin Proteins to Mineral Crystals Through Biochemical and Spectroscopic Assays

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Abstract

Tooth enamel is the hardest tissue in vertebrate animals. Consisting of millions of carbonated hydroxyapatite crystals, this highly mineralized tissue develops from a protein matrix in which amelogenin is the predominant component. The enamel matrix proteins are eventually and completely degraded and removed by proteinases to form mineral-enriched tooth enamel. Identification of the apatite-binding motifs in amelogenin is critical for understanding the amelogenin–crystal interactions and amelogenin–proteinases interactions during tooth enamel biomineralization. A stepwise strategy is introduced to kinetically and quantitatively identify the crystal-binding motifs in amelogenin, including a peptide screening assay, a competitive adsorption assay, and a kinetic-binding assay using amelogenin and gene-engineered amelogenin mutants. A modified enzyme-linked immunosorbent assay on crystal surfaces is also applied to compare binding amounts of amelogenin and its mutants on different planes of apatite crystals. We describe the detailed protocols for these assays and provide the considerations for these experiments in this chapter.

1. INTRODUCTION

Tooth enamel has a unique morphological structure and distinctive mechanical properties, making it different from other mineralized tissues in the human body, such as the bone, dentin, and cementum. Tooth enamel is the hardest tissue known in vertebrates because it is composed mainly of numerous hexagonal carbonated hydroxyapatite (HAp) crystals (Ichijo, Yamashita, & Terashima, 1992; Pergolizzi, Anastasi, Santoro, & Trimarchi, 1995). The crystals form enamel rods and interrods, which weave together and orient in different directions (Pergolizzi et al., 1995; Plate & Hohling, 1994). The crystals in tooth enamel are very thin (20–30 nm). In comparison to the thickness, they are extremely long. Many investigators believe that these crystals span the entire breadth of the tooth enamel, a

distance up to 2.5 mm (Daculsi, Menanteau, Kerebel, & Mitre, 1984; Leblond & Warshawsky, 1979; Nanci, 2003). This thickness/length ratio is equal to that of a 2000-ft. long rope with a diameter of only 1 in.. The unique shapes and organizations of enamel crystals determine the excellent mechanical properties of tooth enamel and also raise a persistent question as to how these enamel crystals form during tooth development.

Mineralized enamel crystals develop from a layer of enamel protein matrix that is predominately amelogenins (>90%), which themselves form special nanostructures to modulate crystal formation through a biomineralization process that is poorly understood. Amelogenins are gradually and completely removed by enamel proteinases, that is, matrix metalloproteinase-20 (MMP-20) and kallikrein 4 (KLK4) to form highly mineralized mature enamel at the completion of maturation stage. The matured enamel matrix in erupted tooth contains less than 2% of the organic components (Bartlett & Simmer, 1999; Fincham, Moradian-Oldak, & Simmer, 1999). In the secretory, transition, and early maturation stages of tooth enamel development, the interactions between crystal, amelogenin proteins, and proteinases dynamically and delicately control not only the growth rate of the crystal but also its growth direction and morphology.

The hexagonal apatite crystals have different surfaces: the (001) face on the top of the rods and (*hk*0) faces on their sides (Fig. 15.1). We found that these distinct surfaces exhibit differential interactions with amelogenin proteins (Habelitz et al., 2004), which may lead to subsequently different interactions between adsorbed amelogenin and proteinases, resulting in a directional growth of HAp crystal in the tooth enamel. Therefore, we need to develop a strategy to identify the crystal-binding motifs in amelogenin and quantitatively compare the amelogenin–crystal interactions for the different faces of apatite crystals.

We designed a series of biochemical approaches to analyze amelogenin–apatite interactions. We have identified the apatite-binding sites of amelogenin by monitoring peptide binding using assays and mass spectrometry as the first screening step. The binding affinities of these identified peptides were further confirmed by their ability to compete with the apatite-binding affinities of full-length amelogenin proteins. The binding kinetics of amelogenin–apatite was then investigated to characterize the putative differences in their protein–crystal interactions between wild-type amelogenin and its mutants.

2. STRATEGY AND RATIONALE

To identify the crystal-binding motifs in amelogenin, we have followed a stepwise strategy. First, we used a peptide screening assay to detect the possible binding sequence(s) in amelogenin. The peptides that were preferentially adsorbed on crystals were identified by matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Second, we preadsorbed the selected peptides onto the crystal surface and then compared their inhibitory effects on the further adsorptions of full-length amelogenin to crystal surfaces. Third, we employed site-directed mutagenesis technique to generate amelogenin mutants carrying deletions within the regions that were indicated to encompass crystal-binding motif(s) and evaluated the impact of the deletions on amelogenin–crystal-binding affinities. The amelogenin mutants containing the different deletions were used to

analyze their binding affinity to crystals. For these protein–mineral assays, we utilized two types of targets: bulk HAp crystals (small size and large surface area) and single fluorapatite (FAP) crystals (large size and small surface area) with special planes exposed. In comparison to FAP crystals, HAp powders offer an advantage of much higher surface-binding area per volume and hence a higher detection sensitivity in screening assays. However, assays based on HAp crystals provide only an ensemble view of amelogenin binding onto both (001) and (*hk*0) apatite crystal surfaces. Discrimination between the distinct crystal surfaces was achieved by using FAP crystals. We note that it was previously demonstrated that structures of FAP and HAp crystals are identical, and hence, FAP is a viable substitute of HAp in amelogenin-binding assays (Habelitz et al., 2004; Tarasevich, Lea, Bernt, Engelhard, & Shaw, 2009).

Combination of all the earlier-described studies allowed us to successfully identify apatite crystal-binding motifs at both N- and C-terminal regions of amelogenin.

3. EXPERIMENTAL COMPONENTS AND CONSIDERATIONS

3.1. Syntheses and identification of amelogenin peptides

Two sets of serial peptides—a total of 25 14-mers and 18 20-mers—were designed to cover the whole human amelogenin sequence (175 residues). For each pair of adjacent peptides, a C-terminal portion of the preceding peptide (7 residues in 14-mer peptide set and 10 residues in 20-mer peptide set) overlapped the N-terminal portion of the subsequent peptide. These peptides were commercially synthesized by a solid-phase peptide synthesis method and purified by reversed-phase high-performance liquid chromatography (Deciphergen Biotechnology, Aurora, CO, USA). The identities of peptides were confirmed by MALDI-TOF-MS.

3.2. Synthesis and analysis of bulk HAp crystals

HAp crystals were synthesized as previously described (Featherstone, Mayer, Driessens, Verbeeck, & Heijligers, 1983) and characterized by X-ray diffraction and Fourier transform infrared spectroscopy (Tanimoto et al., 2008). The HAp crystals were sequentially passed through the meshes at sizes of 30 and then 60 μm . The crystals at the size ranging from 30 to 60 μm were collected. The specific surface areas of the apatite particles were measured by the Brunauer–Emmett–Teller method (Brunauer, Emmett, & Teller, 1938) with a Micromeritics TriStar 3000 (Micromeritics Instrument Corp., Norcross, GA, USA). The surface areas for the various synthetic apatites are similar, ranged within 72 and 80 m^2/g .

3.3. Preparation and identification of single FAP crystal

Single FAP crystals were purchased from Earthlight Gems (Kirkland, WA, USA) and their identities were checked by X-ray diffraction. Diffraction data from single-crystal specimens were collected with a Rigaku rotating anode X-ray source and *R*-axis detector. The crystal form and cell parameters were obtained from analysis of diffraction images, using HKL-2000 or Denzo crystallography software (HKL Research). The determined unit cell parameters, crystal form (trigonal *p*3), lengths ($a = 9.372$, $b = 9.372$, and $c = 6.883$ Å), and angles ($\alpha = 90.000^\circ$, $\beta = 90.000^\circ$, and $\gamma = 120.000^\circ$) were used to search

American Mineralogist Crystal Structure Database (<http://rruff.geo.arizona.edu/AMS/amcsd.php>) with tolerance at 0.004 Å for *a* and *b* and 0.005 Å for *c*. The results unambiguously confirmed that the identity of this material as genuine is FAp crystal.

The single crystals were sectioned into slides with 100 µm thicknesses by using an IsoMet low-speed diamond saw (Buehler, Lake Bluff, IL, USA) along their *c*-axis or *a*- and *b*-axes to expose their (*hk*0) or (001) faces (Fig. 15.1), respectively. The sections were further shaped into squares at a size of 6 × 6 mm using sandpaper of P200 grit size (ISO/FEPA Grit designation). The sections were further sequentially polished by using sandpapers of the grit sizes of P400 and P800 and P1000 and then by utilizing diamond pastes at the sizes of 0.2 and 0.1 µm.

3.4. Expression and purification of recombinant amelogenin

Wild-type full-length amelogenin (rh174) and its mutants were expressed in *Escherichia coli*. The protein was further purified as previously described (Li, Gao, Yan, & DenBesten, 2003).

3.5. Site-directed mutagenesis of amelogenin mutants with deletions of binding motifs

After identification of possible crystal-binding motifs in amelogenin, pairs of primers were designed to flank the cDNA sequences derived from crystal-binding peptide sequences. A PCR-based mutagenesis was performed using a QuikChange® *Lightning* Site-Directed Mutagenesis Kit (Stratagene Inc., La Jolla, CA, USA) according to the manufacturer's manual. The constructs were sequenced to confirm the correct DNA sequence in frame and the presence of the engineered deletions. The expression, extraction, and purification of the amelogenin mutants followed our previous protocols (Li et al., 2003).

4. EXPERIMENTAL APPROACHES

4.1. Identification of specific binding sequences by peptide screening

The studies employed single FAp sections with either (001) or (*hk*0) plane exposed as binding substrates. The FAp sections with different surfaces exposed were incubated with either a mixture of several different peptides or a single peptide. The adsorbed species were sequentially eluted with 10% and then 50% trifluoroacetic acid (TFA) and analyzed by MALDI-TOF-MS. The following generic protocol describes a typical screening procedure using FAp sections. A similar protocol with minor modifications was also used for studies in which bulk HAP crystal powder served as the substrate.

4.1.1—Prelubricated 1.7 ml microcentrifuge tubes (Corning Incorporated, NY, USA) are used to prevent the adhesion of peptides to the tube, which would interfere with the binding amounts of peptides during the assay.

4.1.2—The (*hk*0) faces of FAp sections with (001) predominantly exposed and the (001) faces of FAp sections with (*hk*0) predominantly exposed are carefully coated by a layer of clear nail polish and air dried. Our preexperiments have confirmed that the amounts of the peptides or proteins adsorbed on the nail polish-coated area are negligible (data not shown).

4.1.3—Each FAp section is placed vertically in the prelubricated microcentrifuge tube to double their surface areas of binding. This position of FAp is used for all the following steps.

4.1.4—The individual peptide or a mixture of different peptides is dissolved in Millipore water at a concentration of 5 μ M. Then, 300 μ l of peptide solution is added to the tubes to completely immerse the FAp. The peptide–crystal interactions are performed at room temperature for 5 h with gentle shaking on a platform of a horizontally rotating shaker at 50 rotations per minute.

4.1.5—After incubation, the FAp is transferred to a fresh prelubricated microcentrifuge tube and washed three times with Tris buffer (10 mM Tris–HCl, pH 7.4) with gentle shaking, 10 min per wash.

4.1.6—Then, FAp is taken out of the tube and any remaining solution droplets are moved toward one of the corners and carefully removed with a KimWipes tissue.

4.1.7—The FAp is transferred to a new prelubricated tube containing 300 μ l of 10% TFA to elute the peptides. After gentle shaking for 30 min, FAp is transferred to another tube for a second step of peptide elution with 50% TFA using the same procedure.

4.1.8—Both 10% and 50% TFA elutes are lyophilized to dryness and resuspended in 10 μ l Millipore water.

4.1.9—These eluted peptides are desalted by using a C18 ZipTip (Millipore, Billerica, MA, USA) before MALDI-TOF-MS analyses.

4.1.10—The desalted samples are mixed at 1:1 volume ratio with 5 mg/ml α -cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1%TFA matrix and spotted onto a MALDI stainless steel target plate.

4.1.11—MALDI-TOF-MS experiments are carried out on a 4800 MALDI TOF/TOF Analyzer (AB Sciex, Foster City, CA, USA) at the UCSF Sandler–Moore Mass Spectrometry Core Facility. The samples are ionized with Nd:YAG laser in the reflector (m/z 800–6000) positive-ion mode.

4.1.12—External optimized calibration utilizes OptiPlate software (AB Sciex, Foster City, CA, USA) and is based on four peptide standards within m/z 1290–3800 (angiotensin I and three ACTH clips: 1–17, 18–30, and 7–38) for reflector data.

4.1.13—Spectra are processed with Data Explorer 4.0 software (AB Sciex) by performing baseline adjustment, noise filtering, and deisotoping.

Figure 15.2 shows an example of mass spectrometric data that allowed us to identify peptide P9 for which the residues lie within N-terminal portion of full-length human amelogenin sequence (h175). The MS data demonstrated that the P9 peptide can bind to the (*hk*0) face of

HAp crystals and can be eluted with 50% TFA. This peptide can also be eluted out by 10% TFA, albeit with much lower abundance (data not shown).

4.2. Confirmation of the crystal-binding motifs in amelogenin by competitive adsorption assays

We used a peptide competition strategy to further confirm the amelogenin-binding motifs to apatite crystals by utilizing bulk HAp powders. Following protocol describes the details of this assay. The strategy was to pretreat the crystal surfaces with large excess of peptides and then analyze their abilities to inhibit the further binding of full-length amelogenin. The amount of bound amelogenin was measured and compared to the control conditions of protein binding to a “native” crystal surface. The significant decrease in binding amounts after pretreatment with a given peptide would indicate that this peptide likely carries a crystal-binding motif.

4.2.1—The HAp powders are suspended in Tris–HCl buffer (10 mM Tris at pH 7.4) at concentration of 20 mg/ml.

4.2.2—After mixing by pipetting up and down, 5 μ l aliquots of HAp suspension (100 μ g) are quickly transferred into 1.7 ml prelubricated microcentrifuge tubes.

4.2.3—For the experimental groups, 100 μ l of peptide at concentration of 5 μ M is added into each tube containing HAp powders. The control is treated with the same amount of Tris–HCl buffer without any peptides. The reactions are incubated at room temperature for 1 h with constant tube vortexing to maintain HAp powder in suspension.

4.2.4—Tubes are centrifuged at $5000 \times g$ for 10 min at room temperature and gel-loading tips are used to carefully remove the supernatants.

4.2.5—The pellets are washed with Tris–HCl buffer three times by centrifugation and resuspension.

4.2.6—After washing, the pellets are resuspended in 100 μ l of amelogenin solution (500 μ g/ml). Amelogenin is previously prepared by dissolving in Millipore water and its pH is adjusted to 7.4 with 10 mM Tris–HCl at pH 9.0. The undissolved particles are removed by centrifugation at $5000 \times g$ for 10 min.

4.2.7—The reactions are incubated at room temperature with vortexing.

4.2.8—The reactions are centrifuged at $5000 \times g$ for 10 min. The supernatants are transferred to new tubes.

4.2.9—The protein concentrations in supernatants are measured by Bradford protein assay (Bio-Rad, Hercules, CA, USA). The differences from the original concentration (500 μ g/ml) will be used to calculate the protein binding amounts.

Using this approach, the binding motifs on specific planes of HAp crystals were further confirmed as shown in Fig. 15.3. The data indicate that the pre-treatment of P9 peptide reduces one-third of the binding of full-length amelogenin to HAp crystal powders as compared to untreated crystal samples.

4.3. Comparison of binding amounts of amelogenin and its mutants on different planes of apatite crystals by enzymelinked immunosorbent assay

To confirm the binding domains of amelogenin to different crystal planes, the binding sequences discovered in the previous studies were used to prepare mutated amelogenins with different deletions. All these amelogenin mutants were expressed and purified using the methods as described previously (Le, Gochin, Featherstone, Li, & DenBesten, 2006; Li et al., 2003).

Because the area of the FAp sections is relatively small, the amount of protein bound onto a certain plane is too small to reliably measure using regular protein assays. A system that combines enzyme-linked immunosorbent assay (ELISA) and avidin–biotin complex (ABC) was developed to quantify the amelogenin and its mutants adsorbed on the apatite crystal surfaces. The detailed steps of the experiments are described in the following protocol:

4.3.1—Single FAp sections are prepared as described in Section 3.3 and step 4.1.2. The exposed surfaces are scanned and the images are analyzed by ImageJ software (<http://rsb.info.nih.gov/ij/>) to determine their surface areas. Each group is analyzed in triplicate for statistical analysis.

4.3.2—FAp is prepared as described in step 4.1.2 and placed vertically in a 1.7-ml prelubricated tube containing 300 μ l of amelogenin (500 ng/ μ l). The protein–crystal interactions are performed at room temperature overnight on a platform of a horizontally rotating shaker at 50 rotations per minute.

4.3.3—After incubation, FAp is transferred to a new tube and unbound proteins are washed away in 1 ml PBS for 10 min (three times) with similar shaking.

4.3.4—One milliliter of blocking buffer (PBS containing 1% BSA) is added to each tube to prevent the nonspecific binding of antibodies and the mixture is incubated at room temperature for 1 h.

4.3.5—After blocking, the FAp sections are washed by PBS with 0.05% Tween-20 (3×10 min) with shaking. Remove the solution by aspiration.

4.3.6—Then, FAp samples are incubated with rabbit amelogenin polyclonal antibody at a dilution of 1:1000 in blocking buffer for 1 h at room temperature on the shaker.

4.3.7—Wash step is repeated as described in step 4.3.5.

4.3.8—Three hundred microliters of biotinylated secondary antibody against rabbit IgG (Dako, Carpinteria, CA, USA) at dilution of 1:1000 in blocking buffer is applied into each tube and incubated for another hour under the same condition as previously mentioned.

4.3.9—Washing step described in step 4.3.5 is repeated.

4.3.10—The FAp is incubated with 300 μ l of biotin-conjugated streptavidin–alkaline phosphatase (ABC, Dako), which is diluted at 1:1000 in PBS immediately before use.

4.3.11—After 1 h incubation at room temperature, repeat wash step as step 4.3.5.

4.3.12—During the last washing, the *p*-nitrophenyl phosphate (*p*NPP) substrate solution is freshly prepared by dissolving one *p*NPP tablet (Sigma-Aldrich, St. Louis, MO, USA) and one Tris buffer tablet in 5 ml Millipore water.

4.3.13—Three hundred microliters of *p*NPP substrate mixture is added to each tube with FAp and incubated in darkness at room temperature for 30 min.

4.3.14—The FAp is quickly removed from the tubes. The reaction solution is mixed until the yellow color developed in the tubes appears homogenous. Two hundred microliters of reaction solution is transferred to each well of 96-well transparent microplate.

4.3.15—The plate is immediately read using absorbance plate reader at 405 nm in a spectrometer (Molecular Devices, CA, USA).

The results in Fig. 15.4 show that (1) amelogenins, both the full-length and its mutant (P9 deletion), bind to (001) and (*hk*0) faces of FAp crystals at different levels; (2) full-length amelogenin binds at a higher level than a mutant carrying P9 deletion; and (3) P9 deletion affects amelogenin binding to the (*hk*0) face of FAp to a larger extent than to the (001) surface.

4.4. Kinetic analyses of adsorption of amelogenin to HAp crystals

The kinetic and equilibrium binding character of amelogenin adsorption onto HAp crystals can be assessed by measuring amelogenin capture as a function of amelogenin concentration and incubation time. The caveat here is that one of the reactants is a solid phase: the exposed crystal surface and surface density of HAp molecules can be used to determine an apparent concentration of HAp in the vortexed suspension.

4.4.1—HAp crystal powder is prepared as described in step 4.2.1. After thoroughly mixing, 2.5 μ l of HAp is aliquoted to each 1.7-ml prelubricated tube. The tubes are centrifuged at $1000 \times g$ for 10 min and the supernatant is removed. Please note that the quantity and fineness of HAp crystal powder will significantly affect the measured amelogenin binding rate and response.

4.4.2—Similar to step 4.2.6, amelogenin solution is prepared at three different concentrations (48, 24, and 12 nM). Three is the minimal number of sampling

concentrations for generating a binding isotherm. Several more concentrations may be included to improve the accuracy of projected binding saturation.

4.4.3—The HAp pellets are resuspended in amelogenin solution with the three different concentrations. Triplicate samples for each group are used to reduce errors.

4.4.4—The mixture is incubated at room temperature with gentle vortexing. Vortexing speed and consistency are important for generating reproducible amelogenin adsorption to the HAp suspension. Therefore, the same vortex apparatus should be used for both test and control groups to ensure reliable comparisons.

4.4.5—The samples collected at four time points (0, 2, 8, and 16 min) are centrifuged at $1000 \times g$ for 10 min.

4.4.6—The supernatant is transferred to a new tube and the protein concentration is measured by Bradford assay.

4.4.7—The amounts of amelogenin bound to HAp at each time point are calculated by comparing the protein concentrations before and after binding.

4.4.8—The binding kinetics is analyzed by Prism 6 (GraphPad, La Jolla, CA, USA). The nonlinear regression is used for curve fitting the data to the equations of *association kinetics at two or more concentrations of ligand*.

Figure 15.5 shows the kinetic fitting of full-length amelogenin. The kinetic parameters from global fitting are $K_d = 4.968 \times 10^8 M$, $B_{\max} = 43.73 \pm 7.028$, $K_{\text{on}} = 7.6952 \times 10^{0.06} \pm 1.053 \times 10^6 \text{ s}^{-1}$, and $K_{\text{off}} = 0.3821 \pm 0.048 \text{ s}^{-1} M^{-1}$.

5. DATA HANDLING AND PROCESSING

All quantitative data are statistically processed for their significance by a student *t*-test and/or ANOVA.

6. SUMMARY

A stepwise protocol was developed to identify the binding of motifs in amelogenin to apatite crystals and to compare the difference in their binding on (001) and (*hk*0) faces of the apatite crystals. The assays included an over-lapping peptide screening, peptide competitive adsorption assay, and ELISA on peptide surfaces using amelogenin protein and its mutants in which binding motifs were deleted. The apatite-binding motifs were identified at both amino- and carboxy-termini of amelogenin using this stepwise strategy.

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Figure 15. 1.

FAp single-crystal sections with different faces exposed. Left panel, section with $(hk0)$ crystal face exposed; right panel, section with (001) face exposed. The shaded parts indicate the sections.

Figure 15. 2. MALDI-TOF mass spectra of amelogenin peptides not bound (A) and bound (B) to the (*hk0*) face of the FAp crystals. Peptide P9 is one of the peptides that was eluted out by 50% TFA from the (*hk0*) face after binding (B).

Figure 15. 3.

Reduced binding amounts of full-length amelogenin in peptide competition assays. The percentages are calculated from the comparisons of binding amounts in samples pretreated by peptides (P2, P9, P13, P25, and P28) to untreated samples. ** $P < 0.01$, * $P < 0.05$.

Figure 15. 4.

The effects of mutagenesis of binding motif P9 deletion on amelogenin mutant adsorptions to different faces of FAp crystals. (A) Comparison of adsorption of amelogenin and P9 deletion mutant between different faces of FAp crystals. (B) Different adsorptions between amelogenin and P9 deletion mutants on either (001) or (*hk0*) face of FAp crystals.

Figure 15. 5.
Global dynamic fitting curves of full-length amelogenin binding to hydroxy-apatite crystals.