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Aggregation of 20 kDa Porcine Amelogenin in Solution : FTIR and AFM Analysis of the Accompanying Protein-conformational and Morphological Changes

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Abstract : This study using Fourier transform infrared spectroscopy (FTIR) and atomic force microscopy (AFM) was undertaken to further elucidate the sequential alterations of amelogenin conformation and morphology in the process of protein aggregation. Porcine 148-residue amelogenin (called hereafter the 20 kDa amelogenin), which is the major matrix constituent in pig secretory enamel, was used as a model molecule. The results verified that the protein molecules in a monomeric state at 0°C have multiple conformational elements, whereas most of the structural elements become indiscernible in the progression of protein aggregation, except for the β -strand presumed to exist at the N-terminal region. The observations by AFM confirmed that the self-assembly process of the protein in aqueous milieu generates semi-spherical structures of around 20–30 nm in diameter. The overall results support the theory that the programmed extracellular processing of amelogenins is directly mediated by the conserved primary structure and the resulting supramolecular aggregates.

抄録：本研究では、フーリエ変換赤外吸収法（FTIR）と原子間力顕微鏡（AFM）を用いて、アメロゲニンが溶液内で分子間会合体を形成する過程に生じる構造・形態変化を調べた。観察対象には、ブタ基質形成期エナメル質の主要な基質タンパクである 20 kDa アメロゲニンを選んだ。アメロゲニンの FTIR スペクトル解析から、溶液相で単体分子として存在する条件下（0°C）では二次構造を示すピーク成分が分離されたが、溶液温度の上昇に伴い、会合体が形成される過程で多くの二次構造要素は不明瞭となった。ただし、N 末端領域に存在すると推定される β -シートは分離して観察された。溶液内で形成された 20 kDa アメロゲニンの分子会合体は、その AFM 像では 20–30 nm 径の球体として特徴づけられた。今回の実験結果から、細胞外でのアメロゲニンの酵素切断による低分子化現象は、タンパク分子の一次構造の特質に基づく分子間会合による制御を受けていることが示唆された。

Introduction

Tooth enamel is the hardest tissue in mammals and is composed of the largest calcium apatite crystals in the body. Marked advances have been made in

elucidating the biochemistry and molecular biology of enamel matrix proteins and related genes : We now know that a group of matrix proteins (e.g., amelogenins, ameloblastin, enamelin) and proteases are responsible for the regulation of enamel mineralization during amelogenesis^{1–3}). Amelogenins are the

most abundant enamel proteins, comprising of more than 90% of the proteins secreted by ameloblasts. Sequence comparison of mammalian amelogenins shows their striking homology and conserved domains⁴). A current search for homologies to amelogenin primary structures within the protein sequence data-bases reveals no related structures, supporting the theory of a unique primary structure and functions in this group of proteins⁵). Recognizing that sequence determines structure and structure determines function, much interest has been directed in defining the amelogenin structure at the secondary and tertiary levels. To date, since the crystallization of amelogenin suitable for X-ray crystallographic analysis has not yet been achieved, our understanding of amelogenin structure still remains fragmental. On the basis of the unique properties of amelogenins, i.e., the formation of reversibly equilibrating aggregates in aqueous milieu⁶⁻⁸), it is speculated that, *in vivo*, the matrix exists as a hydrated, three-dimensional gel structure. Unfortunately, protein aggregation phenomena are difficult in most biochemical or molecular biological approaches, and so much remains to be learned about the mechanism of amelogenin aggregation in relation to their structural motifs. The current work using Fourier transform infrared spectroscopy (FTIR) and atomic force microscopy (AFM) was undertaken to gain baseline information about the sequential alterations of amelogenin conformation and morphology in the process of protein aggregation.

Materials and Methods

1. Enamel proteins

The 148-residue amelogenin⁹) (called hereafter the 20 kDa amelogenin) was isolated and purified from pig secretory enamel as reported previously¹⁰). Briefly, tooth germs of lower permanent incisors were obtained from 5 to 6-month-old slaughtered piglets. The tooth germ was dissected out of the bones and the covering soft tissues were removed. The collected secretory enamel was homogenized in 160 mM NaCl and the suspension was separated by centrifugation. The sediment was dissolved completely with 0.5 M acetic acid and the proteins in the acid solution were

desalted and concentrated through an ultrafiltration membrane (YM-2, M. W. cut-off 3 kDa, Amicon). The proteins were separated on a Sephadex G-100 column equilibrated with 50 mM carbonate buffer (pH 10.8). The fractions corresponding to the 20 kDa amelogenin were pooled, and further purified by recycling on the same column. All procedures used for the extraction and purification of 20 kDa amelogenin were conducted at 4°C. Following chromatography, the fractionated sample was desalted and concentrated using ultrafiltration membranes (YM-5, cut-off 5 kDa). Finally, the protein, concentrated in acetic acid, was freeze-dried and stored at -30°C until used. The purity of each separated sample was verified by electrophoreses in the presence of sodium dodecyl sulfate (SDS) or urea, analysis of the amino acid composition, and the determination of the N- and C-terminal residues. Most of these analytical results have been reported elsewhere^{10,11}).

2. Preparation of protein solution

Immediately before use in experiments, a weighed amount of protein sample (typically, 1 mg) was mixed with cold deionized water to yield 1 mg/ml protein solution. Some amounts of the acetate (used as solvent for protein isolation) still remained on the freeze-dried samples; thus, the resulting protein solution was about 5 mM with respect to acetate and the pH value was around 5.3. Under these conditions, the amelogenin protein was solubilized without any visible clouding in the solution. By increasing the pH, ionic strength, and/or temperature of the protein solution, the 20 kDa amelogenin began to precipitate, the protein solution becoming turbid. The pH values of the various solutions were adjusted at specified values by adding minute volumes of NaOH. Solution temperature was controlled by the use of a thermal-jacketed specimen cell described below.

3. Measurement of FTIR spectra of the amelogenin protein in solution

The wavenumbers defined in FTIR spectra have been associated, theoretically and empirically, with the specific structural features of proteins including helix, β -sheet, β -turn, and other or undefined secon-

dary structure^{12–14}). Spectra of the 20 kDa amelogenin in solution were recorded using an FTIR instrument (Perkin-Elmer 1600) in conjunction with a one-mL, multiple internal reflection cell having a ZnSe crystal with 45 degrees incidence angle, which allows convenient and rapid acquisition of the data. The crystal was sealed within a demountable specimen cell by means of a rubber O ring. Circulating water from a water bath was continuously pumped through an attached thermal jacket to maintain the protein solution at specified temperatures as shown in Table 1. The sample temperature was monitored by a digital reading thermocouple. The resolution used was 4 cm⁻¹ and 128 scans were taken for each recording of the spectrum. All sample spectra were normalized against a dry-cell background of 128 scans collected at the desired working temperature. Immediately after background collection the spectra of the buffer and then of the sample in buffer were obtained, using the same scans for each collection. The deconvolution of the experimental spectra was accomplished by the use of the program Spectra Calc (Galactic Industries Co.). Data analysis consisted of digital subtraction of the appropriate solvent spectrum from the spectrum of the protein in solution. Attention was directed to the Amide I band observed in the spectral region of 1,690–1,620 cm⁻¹, which is generated primarily from the stretching vibrations of the peptide carbonyl group. Spectral characteristics in these regions can be used to distinguish the relative proportions of α -helix, β -sheet, β -turns, and disordered structures within the protein (see the footnote of Table 1).

4. Atomic force microscopic observation of porcine amelogenin in solution

AFM imaging permits the direct visualization of protein morphology in a hydrated environment. In the current work, a mica slip was placed on the bottom of a specimen holder, which was subsequently filled with 0.5 mL of protein solution. Protein molecules adsorbed onto the mica surface were imaged *in situ* using a NanoScope II AFM (Digital Instruments) equipped with a 16×16 μ m scanner and 100 μ m Si₃N₄ cantilevers. Typical forces applied while imaging ranged from 10⁻⁸ to 10⁻¹⁰N. In preliminary observations,

protein images were recorded at a variety of magnification and rotations to insure that the probe was not influencing the configurations of the molecules. The results confirmed that the protein molecules were stable when precipitated on the mica surface. All observations were made at room temperature (around 25°C) because it was not feasible to accurately control the temperatures of a protein solution placed in an open specimen container. In order to modulate the aggregation state of protein molecules in solution, we adopted two approaches: (1) changing protein concentrations between 0.01 and 1 mg/mL, or (2) adjusting the pH of protein solution up to 7.0 with the addition of NaOH. Three-dimensional color graphics were generated on the computer using the installed Nano Scope Software. Diameters of protein particles or aggregates were determined for over 100 such structures from the AFM image-data files using a precalibrated dimensioning tool.

Results

1. Changes in FTIR spectral features of the 20 kDa amelogenin as a function of solution temperature

When FTIR spectra were recorded from aqueous protein samples at the low concentration (around 1 mg/mL), H₂O spectrum was the overwhelming contributor to the experimental spectrum (Fig. 1 A). However, even from such a dilute protein solution, the protein absorption bands in the 1,600–1,700 cm⁻¹ region were discernible and, after subtraction, were successfully separated from the strong water adsorption bands. Figure 2 shows representative spectral changes as a function of solution temperature. At 0°C, the Amide I contour exhibited a substantial complexity and the curve-fitting results disclosed the involvement of at least 10 peak components. The wavenumbers of the separated peaks and their relative band areas are shown in the Table 1. Each band was assumed to be associated with a particular structural element according to the assignments shown in the footnote of the Table 1. Changes in the shape and relative intensities were apparent as a function of temperature. Most remarkably, the peak maximum of

Fig. 1 Comparison of the relative intensities of Amide I and the water absorption bands.

Protein spectrum (C) was obtained by subtracting a solvent spectrum (B) from an experimental spectrum of protein solution sample (A). The numerals represent the frequency in cm^{-1} of each band at maximum intensity.

Fig. 2 Detailed comparison of deconvoluted Amide I absorption bands of the 20 kDa amelogenin at 1 mg/ml in deionized water at different temperatures.

As indicated by the overlapping peak-profiles in each spectrum, the calculated spectrum, which was obtained by a sum of individual peak-components, in general fits well to the experimental one. Temperatures adjusted for measuring experimental spectra were (A) 0°C, (B) 6°C, and (C) 10°C. Note the appearance of a broad peak component around $1,648\text{ cm}^{-1}$ (see an arrow in spectrum C) with increasing solution temperatures.

the experimental spectra recorded at 6°C and higher temperatures shifted to the low wavenumber region : the peak components in the range of $1,652\text{--}1,676$

cm^{-1} (supporting the existence of helix, turns and bends) were most intensive at the lower temperatures (0 and 6°C), whereas the broad absorption at $1,648$

Table 1 Results of FTIR spectral analysis of Amide I bands measured for 20 kDa porcine amelogenin in solution

Temperature (°C)	Wavenumbers (cm ⁻¹) and relative peak-areas							
0	1,606 [95]	1,620 [30]	1,630 [77]	1,642 [74]	1,652 [100]	1,662 [37]	1,676 [88]	1,688 [17]
6		1,610 [276]	1,618 [18]	1,630 [93]	1,642 [100]	1,650 [100]	1,662 [91]	1,678 [125]
10	1,608 [77]		1,630 [55]	1,648 [100]		1,668 [66]		1,684 [18]
20	1,604 [71]	1,610 [10]	1,624 [15]	1,634 [20]	1,648 [100]		1,672 [36]	1,688 [7]

Peak-areas shown in the bracket are presented as the normalized values in reference to the area of a separated peak at 1,648–1,652 cm⁻¹. Putative secondary structures assigned based on the wavenumbers (cm⁻¹) were : distorted & β -sheet, 1,600–1,620 cm⁻¹ ; extended chains, 1,620–1,640 cm⁻¹ ; unordered, 1,640–1,650 cm⁻¹ ; helix, 1,650–1,660 cm⁻¹ ; turns and bends, 1,660–1,700 cm⁻¹.

cm⁻¹ became prominent at the higher temperatures (10 and 20°C), suggesting an increase in the unordered structure. Of most interest, the bands in the wavenumber range of 1,604–1,610 cm⁻¹ (plausibly assigned to the β -sheet) remained stable throughout the temperature range examined.

2. AFM imaging of aggregation of 20 kDa porcine amelogenin

In aqueous solution at room temperature, the protein molecules above 0.05 mg/ml started to sediment onto the flat mica surface. Aggregates were dispersed and spherical in appearance, although some deformed ellipsoidal particles were also noted (Fig. 3 A). Figure 3 B shows a top view of an isolated spherical body, the diameter of which, measured along the scanning line, was approx. 20 nm (Fig. 3 C). The packing of the spherical aggregates increased as a function of protein concentration, and assemblies of these spheres were observed with diameters greater than 100 nm. Similar alterations in the protein images from singular particles to massive aggregates were also attained by adjusting to neutral pHs.

Discussion

In the last two decades, the conformation of

amelogenins has been studied using both physical methods and predictive strategies. Chou and Fasman analysis suggests that the conserved N-terminal segment (residues 1–40) of the 20 kDa porcine amelogenin contains β -strand and β -turns. One of the distinguishable structural features of amelogenins is the occurrence of multiple tandem (or triple) repeat sequences such as His-His, Pro-Pro, and Gln-Gln ; however, the significance of these repeat motifs at the secondary and tertiary levels remains to be elucidated. Molecular modeling studies suggest that the secondary structure of the marked repetitive sequence (Gln-Pro-Xxx)_n noted for bovine amelogenin is a β -spiral¹⁵⁾. However, no further evidence has been reported to confirm this theory. Currently, it is accepted that proline residue does not merely act as a spacer, but has an important structural role in generating and stabilizing an extended structure ; specifically, a high proportion of proline (one residue in three) precludes any significant α -helical motif. It has also been theorized that a large flat hydrophobic surface of proline may associate with other flat hydrophobic surfaces such as aromatic rings and that the proline-rich motif facilitates rapid and reversible protein-protein interactions¹⁶⁾. In our study utilizing FTIR we found that when the 20 kDa amelogenin was solubilized at 0°C, the protein molecule had multiple conformational

Fig. 3

- (A) A three-dimensional AFM view of the 20 kDa porcine amelogenin aggregates formed in aqueous environment and adsorbed onto a mica surface.
- (B) A top-view of a single spherical body of the protein aggregate. The diameter of the sphere was measured along the horizontal line using a precalibrated dimensioning tool.
- (C) The generated line scanning profile corresponding the sphere staying on the mica plane. The value of the diameter was 28.4 nm.

elements. The monomeric state of the protein under the comparable solution environment was demonstrated in previous studies using low-angle laser light scattering analysis coupled with high performance gel chromatography¹¹⁾. With increasing solution temperature or protein concentrations, the current AFM observation confirmed the general occurrence of spherical supramolecular assemblies of amelogenin molecules in aqueous solutions at room temperature. Fincham *et al.*^{2,8,17)} called these structures “nanospheres”, and theorized that they are comparable to the so-called stippled materials seen *in situ* by transmission electron microscopy. In relation to the conformational changes associated with amelogenin aggregation, it was of note that, whereas most of the spectral details corresponding to discrete conformational elements deteriorated during the progression of protein aggregation due to burying inside the su-

pramolecular aggregates, one spectral element putatively assigned to the β -strand remained relatively stable. At present, no comprehensive explanation is available for the relative stability of the β -strand, which we speculated exists in the N-terminal segment, but this finding supports the hypothesis that the N-terminal segment of amelogenin may possess a more rigid structure in comparison with the core and C-terminal segments, which have been theorized to be flexible¹⁸⁾ or in rapid isotropic (random) motion¹⁹⁾. Moradian-Oldak *et al.*²⁰⁾ theorized that the N-terminal domain is involved in the molecular interactions in the formation of nanospheres; the carboxy-terminal domain contributes to the stability and homogeneity of the nanospheres, preventing their fusion to larger assemblies.

The past conformational studies of the amelogenins entailed X-ray diffraction, nuclear magnetic reso-

nance (NMR), and circular dichroism (CD), as well as the FTIR used in our study. Our previous studies^{11,18)} using CD and NMR showed that : (1) the molecular structure of the intact porcine amelogenin consists of discrete folding units, i.e., the N-terminal, central (core) and C-terminal domains, and that these units have little, if any, interaction with each other. We recently reported a tentative structural model of the 20 kDa amelogenin, which was constructed based on the results of small angle X-ray scattering (SAXS), in conjunction with the molecular modeling analysis²¹⁾. The molecular model predicts an elongated bundle structure consisting of one part of β -sheet and two parts of polyglycine II, interspersed with a "loop" at the amino acids 38 to 45 (Gly-Tyr-Glu-Pro-Met-Gly-Gly-Trp) and a β -turn at amino acids 89 and 90 (Pro-Gly). According to this bundle model of 20 kDa amelogenin monomer, we speculated that the extended molecular surfaces face each other, generating the spherical aggregates as visualized by AFM. The rigid β -sheet conformation in the N-terminal region, suggested by the current FTIR data, may play a pivotal role in mediating the intermolecular association. Notably, SAXS results allowed us to assess the size of individual bundle monomers along their long axis ; the calculated values, depending on the assumed molecular shapes of an extended ellipsoid and rod, are 9.5 and 14.9 nm, respectively (unpublished). Considering that the amelogenin monomer is an extended ellipsoid in shape, the calculated dimension of the monomer seems to fit the radius of the nanosphere determined by AFM. A simple calculation suggests that the single nanosphere of 20 nm diameter, as shown in Fig. 3 B, may comprise around 500 of amelogenin ellipsoids.

The 20 kDa amelogenin becomes most abundant throughout the depth of the secretory enamel^{7,22)}. The apparent stability of this molecule, in sharp contrast to the transient nature of the full-length (i.e., 25 kDa) amelogenin⁹⁾, may be due to buried putative cleavage sites inside the supramolecular aggregates and limited protease accessibility. If the same aggregation model was applicable to the 25 kDa amelogenin, we speculate that the hydrophilic C-terminal segments may align to cover the spherical body, exposing the exter-

nal milieu ; such a quaternary configuration would facilitate the access of proteases to cleavage sites in the hydrophilic external surface. Regarding the functional significance of amelogenin aggregates, we speculated that the supramolecular structures could function to organize the microstructure of the matrix, spacing the initial crystallites at certain distances (>20 nm) and inhibiting early crystal-crystal lateral fusions. Wen *et al.*²³⁾ stated that the hydrophobic interactions among nanospheres and different orders of amelogenin assemblies are important in determining the structural integrity of enamel matrix, which may be responsible for supporting the immature, elongating enamel crystallites during the secretory stage. Regardless of the uncertainties remaining as to the exact molecular state and functional significance, it is now established that the rapid proteolytic transformation, from the full-length hydrophilic molecule to a less transient protein molecule lacking the carboxyl-terminal segment, is the programmed event universally observed in the mammals^{24,25)}.

As emphasized in the literature, a central issue of biomineralization is to understand how the specific biosystem can regulate the nucleation, growth, morphology, and assembly of the inorganic crystals²⁶⁾. Such regulation is achieved, at least in part, by specific processes involving molecular recognition at inorganic-organic interfaces. Obviously, amelogenins and their aggregation products play pivotal roles in enamel mineralization. To fill the current gap of our understanding as to the structure-function relationship of amelogenins and other matrix components, further structural biology investigation of enamel extracellular matrix proteins is required to elucidate the secondary, tertiary, and quaternary structures of amelogenins.

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