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Evidence for Charge Domains on Developing Enamel Crystal Surfaces

ABSTRACT

The control of hydroxyapatite crystal initiation and growth during enamel development is thought to be mediated *via* the proteins of the extracellular matrix. However, the precise nature of these matrix-mineral interactions remains obscure. The aim of the present study was to use a combination of atomic and chemical force microscopy to characterize developing enamel crystal surfaces and to determine their relationship with endogenous enamel matrix protein (amelogenin). The results show regular and discrete domains of various charges or charge densities on the surfaces of hydroxyapatite crystals derived from the maturation stage of enamel development. Binding of amelogenin to individual crystals at physiological pH was seen to be coincident with positively charged surface domains. These domains may therefore provide an instructional template for matrix-mineral interactions. Alternatively, the alternating array of charge on the crystal surfaces may reflect the original relationship with, and influence of, matrix interaction with the crystal surfaces during crystal growth.

KEY WORDS: enamel, crystals, development, amelogenin, AFM.

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INTRODUCTION

Dental enamel is the most extreme example of mammalian biomineralisation. Its physical properties and physiological function are fundamentally related to the composition, orientation, morphology, and disposition of the mineral component within the tissue. Enamel mineral takes the form of crystals of carbonated calcium hydroxyapatite which are larger and more uniform than those found in the mesenchymal skeletal tissues such as dentin and bone (Daculsi and Kerebel, 1978). The control of hydroxyapatite crystal deposition and growth is therefore a central process in enamel development. This control has long been thought to be mediated via the proteins of the developing enamel matrix (Akita *et al.*, 1992; reviewed by Robinson *et al.*, 1995). Precise control mechanisms remain obscure, but interactions between crystal surfaces and specific stereochemical arrays on extracellular matrix proteins have been implicated (Simmer and Fincham, 1995).

During enamel formation, very thin, ribbon-like crystals of hydroxyapatite are initially deposited in a complex extracellular organic matrix, composed primarily of a family of related proteins derived from a single gene—the amelogenins (Fincham and Moradian-Oldak, 1995). These proteins are subject to extensive post-secretory processing, generating a spectrum of discrete components with specific spatial distribution within the developing tissue (Brookes *et al.*, 1995). We have previously demonstrated that failure to remove the organic matrix results in incomplete crystal growth and therefore maturation of the tissue, implying an inhibitory or at least modulating role for these proteins in terms of crystal growth (Robinson *et al.*, 1989, 1992). However, despite a great deal of effort, techniques applied to date have been unable to elucidate the precise behavior of specific matrix components at mineral surfaces where, presumably, any control must be effected.

We have recently used the atomic force microscope (AFM) to provide topological images of hydroxyapatite crystals from developing enamel, revealing putative kink and growth sites which may be important determinants in the mechanisms of crystal growth during enamel biomineralization (Kirkham *et al.*, 1998). AFM is ideally suited for biological imaging, since specimens do not need to be dehydrated, fixed, stained, or coated, and, most importantly, imaging can be carried out under fluids, maintaining the correct biological milieu (Ikai, 1996). In addition, with the use of functionalized AFM tips which are chemically modified by thiol-linked self-assembled monolayers, chemical force microscopy (CFM) (Noy *et al.*, 1997) can be used to provide more specific information about surface charge properties of skeletal mineral crystals and binding strengths of interacting ligands.

The aim of the present study was to use CFM to map the surface charge characteristics of developing enamel crystals and to investigate how this might relate to protein-mineral interactions.

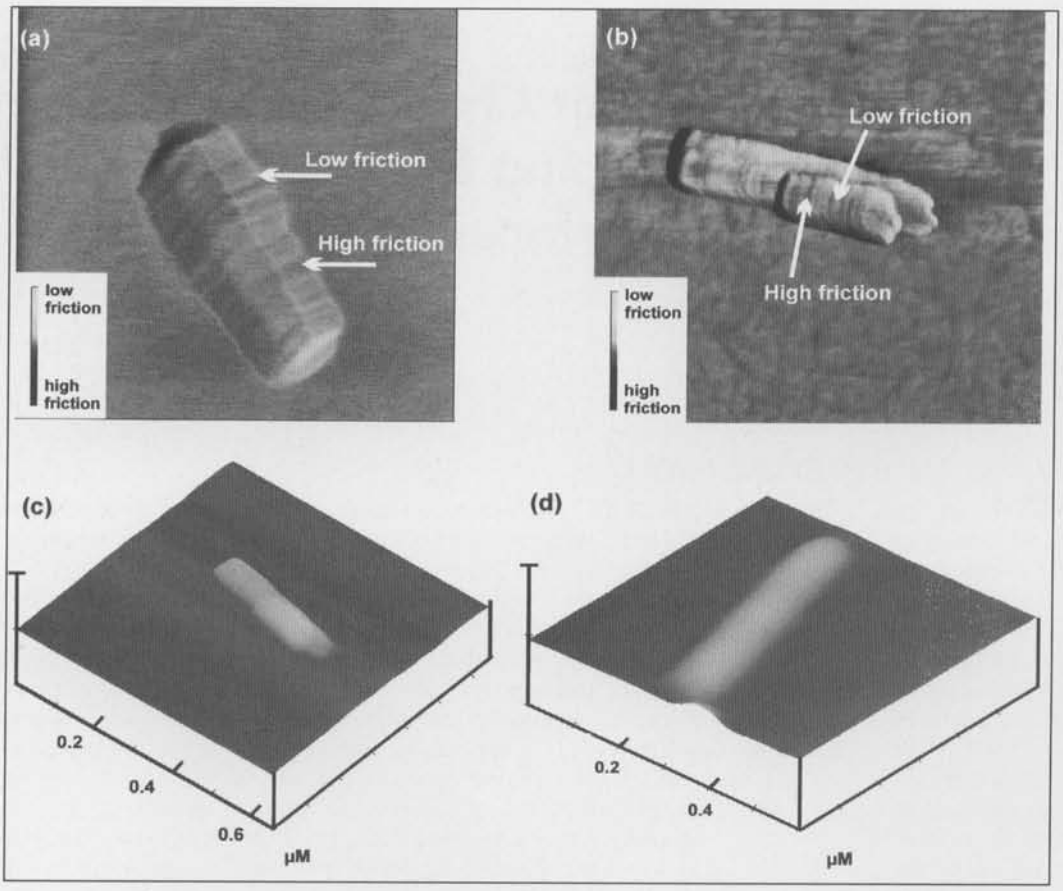

Figure 1. Surface characteristics of hydroxyapatite crystals isolated from the developing enamel of rat incisor teeth, imaged under fluid at pH 7.4. **(a)** CFM image obtained in lateral force mode with a carboxyl-modified tip. Bands of high and low friction, corresponding to alternating charge domains, can be seen. **(b)** CFM image obtained in lateral force mode with an amino-modified tip. Bands of high and low friction can be seen which are a reverse image of those obtained with the carboxyl-modified tips. **(c)** AFM (height) image of (b) above, showing surface topography of crystals. **(d)** Typical AFM (height) image of crystal obtained with a non-modified tip. For both (c) and (d), crystal surfaces were smooth, suggesting that the patterns observed in CFM lateral-force mode were not related to differences in height.

MATERIALS & METHODS

Preparation of Enamel Crystals

Individual crystals, free from all endogenous matrix protein, were prepared from the maturation stage of developing rat incisor enamel as described previously (Robinson *et al.*, 1996; Kirkham *et al.*, 1998).

Chemical Force Microscopy (CFM)

Isolated developing enamel crystals were immobilized on specially prepared substrates as described previously (Zhang *et al.*, 1999). Chemically modified ("functionalized") Si_3N_4 tips were generated by being coated with a 10-nm chromium adhesion layer followed by 100-nm gold (99.99% pure) deposited by thermal evaporation at a constant rate of 0.1 nm/sec. Self-assembled monolayers (SAMs) were then formed by immersion of the tips in solutions containing either 0.1 mM 11-mercaptoundecanoic acid (Aldrich, Poole, UK) or 0.1 mM 11-amino-1-undecanethiol hydrochloride (Dojindo, Tokyo, Japan) in 0.5 mM ethanol for 24 hrs at room temperature. Upon removal from solution, the tips were rinsed thoroughly with absolute ethanol and, finally, distilled water (pH = 7) before use. CFM images with the functionalized tips were obtained under fluids of various pH, by means of the Molecular Imaging™ picoSPM controlled by Nanoscope IIIa electronics (Digital Instruments, Santa Barbara, CA, USA) with a 40 x 40 µm scanner in lateral force mode.

Atomic Force Microscopy (AFM)

AFM images of protein-mineral binding were obtained by means of a Nanoscope IIIa AFM (Digital Instruments) fitted with a flow cell. Enamel crystals were added to freshly cleaved mica as described previously. Recombinant amelogenin (M179, Simmer *et al.*, 1994) was introduced into the flow cell at 50 µg/mL in 20 mM Tris, pH 7.4. Images were obtained by means of standard 200-µm Si_3N_4 cantilevers (Park Scientific Instruments, Northampton, UK) in tapping mode at near-resonance frequencies (9 KHz), with a resonance amplitude of 0.6 V, set point of 0.5 V, and scan rates of 2.5 Hz, with 256 lines taken *per* image.

RESULTS

Chemical Force Microscopy of Maturation-stage Enamel Crystals

We used chemical force microscopy with functionalized tips (both negatively and positively charged) in lateral force mode to interrogate the surfaces of individual maturation-stage crystals from developing enamel in an attempt to identify variations in surface charge. CFM revealed repeating alternating domains of surface charge in the direction of the crystallographic "c" axis (Figs. 1a, 1b), comprising broad bands (from 30 to 50 nm in width) interrupted by narrower domains (approx. 15 nm in width). Carboxylated (negatively charged) and amino-terminated (positively charged) tips produced reciprocal images of each other, suggesting that the banding pattern observed was due to distinct differences in charge, or charge density, at the crystal surface. The corresponding topographic (height) images (Fig. 1c) showed no evidence of surface features which might induce artefacts in the lateral force images.

Atomic Force Microscopy of Crystal-Protein Interactions

Fig. 2 shows the alignment of amelogenin protein about the "c" axis of individual crystals from the maturation stage of developing rat incisor enamel. The protein appeared to be present in the form of spheres, approximately 30 nm in width. The size and form of the spheres were similar to those

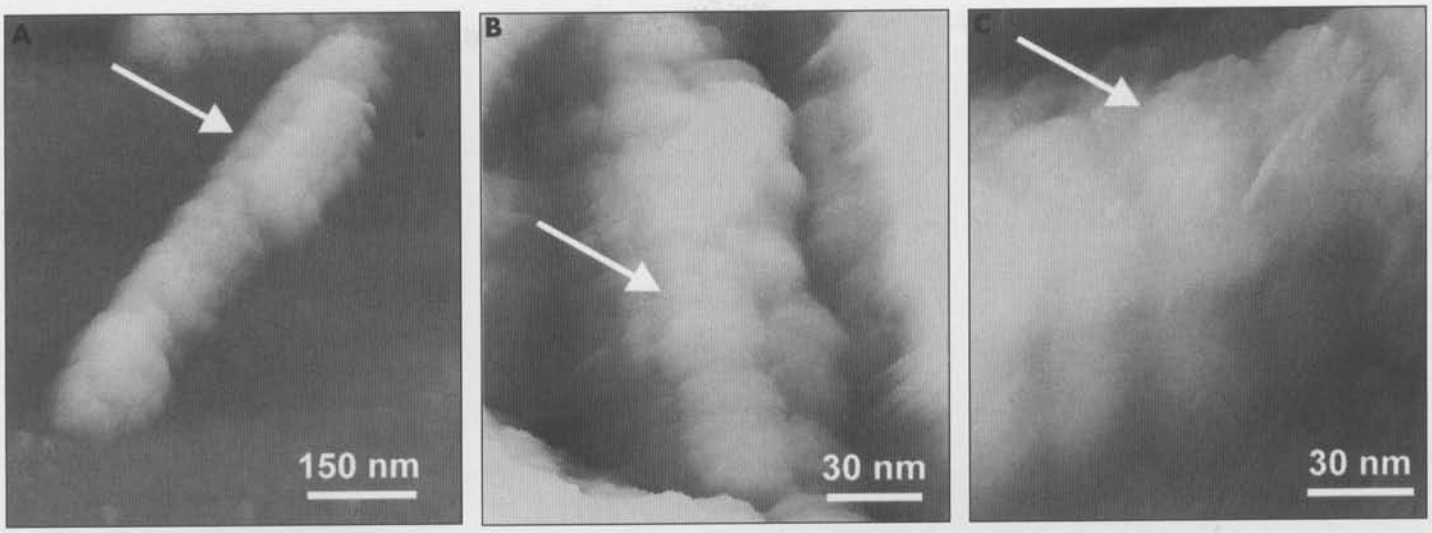

Figure 2. AFM at increasing magnifications **(A-C)** images of hydroxyapatite crystals isolated from the developing enamel of rat incisor teeth following exposure to amelogenin protein. Amelogenin can be seen to have aggregated to form spherical structures (arrowed) which align along the crystal "c" axis corresponding to the positive charge domains seen in CFM.

described previously for amelogenin aggregates or nanospheres (Moradian-Oldak *et al.*, 1994). The periodicity of the spacing of amelogenin aggregates along the crystal "c" axis closely corresponded to that of the positively charged domains revealed by CFM. Desorption of protein by means of increasing concentrations of phosphate buffer confirmed this.

DISCUSSION

In a previous study, we demonstrated that maturation-stage enamel crystals remained in position during AFM scanning in contact mode under fluids when bound to negatively charged substrates (Zhang *et al.*, 1999). Scanning was carried out under simulated enamel fluid at physiological pH, implying an overall positive surface charge for developing enamel crystals *in vivo*.

Previous work, based upon measurements of zeta potential, has generated conflicting results in terms of net surface charge on hydroxyapatite crystal surfaces (reviewed by Chander and Fuerstenau, 1984). However, there are reports of calcium-rich surface layers on biologically derived crystals which are commensurate with the findings described here (Mafe *et al.*, 1996).

The results of the chemical force microscopy reported here are supported by these earlier findings of overall positive charge on the developing crystal surfaces. The contrast in the CFM images arises from a twisting of the AFM cantilever due to lateral frictional forces, presumably arising from attractive or repulsive electrostatic interactions between the charged tip and the substrate (Noy *et al.*, 1995). This suggests that the bands or domains on the crystal surfaces might represent alternating charge polarity or domains of similar polarity but different charge density. If the broader bands are positively charged and the narrower bands negative, then a net positive surface charge would result. Alternatively, both bands may be positive but of differing charge density. Again, this would result in an overall positively charged crystal surface.

The CFM images shown here were obtained under ethanol, which produced the greatest definition and resolution. CFM imaging under physiological solutions is less successful, due to the presence of ions which result in an electric double-layer (Butt *et al.*, 1995; Hillier *et al.*, 1996), obscuring the tip-sample interactions and limiting resolution. The repeating pattern of surface charge density or polarity is previously unreported, and its imaging has been made possible only by the use of chemical force techniques. The pattern has been a consistent finding from many such experiments and with different operators using different preparations of maturation-stage crystals. It is not possible, by these techniques, to determine what groups give rise to the charge variations and how these relate to the hydroxyapatite crystal lattice, if at all, since the AFM is unable to resolve at the atomic level on biological samples due to their inherent surface roughness. One further constraint of the technology is that it is not possible to compare younger crystals derived from the secretory stage of enamel development with the maturation-stage crystals. CFM with lateral force as described here requires that the substrate be smooth so that there is no artefact induced by surface topography. Our previous studies have demonstrated that there is a significant decrease in crystal surface roughness with development (Kirkham *et al.*, 2000). This limits the application of the technique to crystals from the maturation stage, since secretory-stage crystals are too rough. Further investigations are under way with force-mapping techniques to address this problem.

The role of amelogenin in the control of crystal growth has long been debated. Previous reports have suggested an inhibitory role, operating either via direct binding to the crystals, obscuring growth sites, or by provision of a physical (hydrophobic) barrier preventing access of mineral ions to developing crystal surfaces (Aoba and Moreno, 1991; Robinson *et al.*, 1995; Simmer and Fincham, 1995). During enamel development, amelogenin is sequentially degraded to generate a range of lower-molecular-weight processing products which are temporally and spatially restricted and have various degrees of affinity for the mineral phase. In the present study, we used a recombinant full-length amelogenin molecule (M179) which lacks only the N-terminal methionine and phosphorylation at serine-16. The protein appeared to adopt the form of spheres, approximately 30 to 50 nm in diameter, which

closely resemble previously reported "nanosphere" structures observed both *in vitro* (Fincham *et al.*, 1994) and *in vivo* (Robinson *et al.*, 1981). Such structures have been reported to contain > 100 individual amelogenin molecules *in vitro* and are aggregates of the nascent protein (Moradian-Oldak *et al.*, 1994). However, *in vivo*, they probably are comprised of a mixture of parent molecules and their breakdown products. Nascent amelogenin has been previously reported to bind to hydroxyapatite and to enamel crystals (Aoba and Moreno, 1991; Robinson *et al.*, 1997). The association of nanospheres with crystal surfaces observed in the present study demonstrates, for the first time, that the parent protein in its aggregated form binds to developing crystals.

The spatial arrangement of amelogenin aggregates on the crystal surfaces indicates that binding may be according to a spatially pre-determined pattern dictated by surface charge domains on the crystals themselves. These charge domains are therefore clearly obvious candidates for a role in matrix-mineral interactions.

This arrangement of amelogenin along the crystal surfaces would also explain the observed preferential crystal growth along the "c" axis and apparent inhibition of crystal growth in width and thickness dimensions in the presence of these proteins, particularly in the newly secreted tissue. Amelogenin is sequentially degraded with time by temporally restricted proteolytic enzymes (Bartlett *et al.*, 1996; Simmer *et al.*, 1998), presumably permitting subsequent controlled lateral growth of the crystals to occur. It is unlikely that amelogenin would bind to the growing end of the crystal in the same way as seen in these studies, since this would be a much smaller surface which could not have the same spatial patterning of charge (the ends of the crystals in our experiments do not represent the true ends of the crystal *in vivo*).

The repeating patterns of crystal surface charge may therefore be central in providing an instructional template for interactions with the matrix. Alternatively, the observed alternation of charge bands on the crystal surface may be a developmental remnant arising from crystal elongation as the parent amelogenin aggregate is processed and removed, reflecting the original relationship with and influence of the initial matrix structures on the growing crystal.

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