Physico-chemical properties of crystal surfaces in matrix–mineral interactions during mammalian biomineralisation

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Abstract

Surfaces of developing enamel crystals were shown to comprise of alternating domains of positive and less positive (perhaps even negative) charge density which directly bind a number of matrix proteins via electrostatic interactions. Studies using synthetic mineral crystals demonstrated stereo-specific docking of charged residues with crystal lattice sites. Preferential binding of matrix proteins to specific crystal faces related to interfacial hydrophobicity, was shown to control crystal habit. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Biomineralisation—the controlled deposition and regulated growth of minerals in biological systems—occurs throughout nature, from prokaryotes to the skeletal tissues of the higher mammals [1]. The type, form and function of biominerals are extremely varied but all share a common theme in the exquisite control mechanisms which determine crystal disposition and morphology, culminating in the production of biomaterials with often complex architecture and specific biological function. Elucidation of the principles governing these mechanisms would permit design of biomimetic materials and potential therapeutics with applications not only in the biomedical sciences, but also in the wider scientific arena.

A great deal of work has been directed towards determination of the mechanisms of matrix mediated biomineralisation in the mammalian skeletal tissues and with the advent of new analytical tools, advances have been made in our understanding of the nature of matrix–mineral interactions. The purpose of this review is to consider the recent evidence describing the basis of the interaction between matrix proteins and mineral crystals in the skeletal tissues, as the basis for the biological control of crystal growth and morphology during mammalian biomineralisation.

The principle biomineral in mammalian skeletal tissues is a substituted calcium hydroxyapatite
[Ca₁₀(PO₄)₆OH₂] (HAP). Carbonate is the major heteroionic substituent [2] but fluoride, magnesium and other trace elements are also present in significant quantities. Crystal chemistry and morphology vary between the skeletal tissues, with the crystals of the collagenous mesenchymal tissues (bone, dentine, cementum) being much smaller, irregular and more highly substituted than the crystals of dental enamel, which is epithelial in origin. Enamel is the most extreme example of mammalian biomineralisation, with > 97% of the mature tissue comprising of HAP crystals. In contrast with the mesenchymal skeletal tissues, the organic matrix of enamel is ephemeral, being degraded and subsequently lost as the tissue matures and secondary crystal growth occurs [3*].

The size and form of the crystals within mineralised tissues reflect the controls and constraints imposed during tissue morphogenesis [3*,4]. Given that any interaction between matrix macromolecules and mineral crystals must be mediated via association with the crystal surfaces, physico-chemical characterisation of crystal surfaces is a key requirement to the understanding of the matrix–mineral relationship. In this respect, definition of the ‘surface’ itself becomes an issue since in aqueous solutions, the liquid double layer immediately adjacent to the crystal solid is different from the liquid surrounding it and the surface of the underlying solid is different from that of the bulk [5]. It is therefore important to remember that much of the characterisation of HAP facial surfaces may not relate directly to the inner bulk of the solid itself.

Investigations to define skeletal crystal surfaces are a challenge since extraction and isolation of biological crystals inevitably changes their surface characteristics. Subsequent determination of protein–mineral interactions using biological crystals is also difficult for similar reasons and until recently, very little work was carried out under physiological conditions. Many workers have therefore used synthetic HAP or octacalcium phosphate (OCP) as a substitute for natural skeletal mineral. OCP has been suggested to be a precursor for HAP in biological systems as HAP twinning occurs on the 010 face of OCP [6], and recent reports have described the co-existence of both mineral phases within single crystals of dentine [7]. In addition, the transition layer between bulk HAP and the aqueous phase surrounding the crystal surface is OCP-like [5], and both salts share similar surface structure and properties. However, it is important to realise that synthetic minerals may be entirely different in their surface chemistry to those found in vivo.

2. Physical characteristics of crystal surfaces

Developing dental enamel is possibly the only mineralised tissue where individual crystals can be isolated relatively easily. Crystals from developing enamel were therefore used in the first experiments describing the surface characteristics of biological crystals using atomic force microscopy (AFM) and chemical force microscopy (CFM) [8*,9*,10**]. AFM studies revealed that the topography of enamel crystals exhibited surface rugosities of the order of size of a single HAP unit cell which corresponded closely with growth sites described for synthetic HAP crystals [11**] (similar features have also been seen on single crystals isolated from bone, see Fig. 1). With increasing crystal maturation, these surface features decreased in density. This surface ‘smoothing’ effect was also seen when growth of immature crystals was determined in vitro (see Fig. 2). The data potentially reflect a change in crystal growth modality at different stages of enamel crystal development, from a polynuclear ('birth and spread') model to spiral growth during maturation. This, in turn, would be expected to be related to the degree of supersaturation with respect to the growing

Fig. 1. AFM 3-D rendered image showing a single crystal isolated from rat tibial bone. The dotted line shows the sampling location of the adjacent line scan (obtained from the original height image). Vertical distances between surface steps (possibly indicative of spiral growth) are delineated by the coloured arrows.
mineral phase in the surrounding milieu, as polynuclear growth is associated with conditions of high supersaturation while spiral growth is associated with lower levels [12]. Introduction of fluoride ions significantly increased the number of putative growth sites on the crystal surfaces [13]. This may reflect a shift in prevalent growth modalities associated with supersaturation and precipitation on the surface of a different mineral phase.

Growth sites on crystal surfaces are obvious candidates for protein binding if inhibition of growth is to be effected. During enamel development, the crystals are laid down in a protein-rich matrix and remain as long, thin, ribbon-like structures until secondary crystal growth in the $a$-$b$ and $b$-$c$ directions occurs in association with matrix degradation and removal [3]. Inhibition of lateral growth and control of crystal morphology are therefore presumably determined by protein binding, though the physico-chemical characteristics of the mineral itself cannot be ignored (for example, growth in the $c$-axis is thermodynamically favoured and incorporation of carbonate into stoichiometric HAP results in a change in crystal morphology from needles to plates, and may have important implications in considering the highly substituted crystals of bone and dentine) [14,15]. The relative size of mineral-binding proteins compared with the individual crystals is such that very few proteins would be needed to effect control, and recent evidence suggests that less than 1% of all possible growth sites are actually active during crystal growth [12]. The increased crystal surface area associated with fluoride substitution may be important in respect of increasing available binding sites for protein. Increased surface nanotopography was found to promote adsorption of vitronectin on to HAP [16] and immature HAP crystals with irregular habits adsorbed greater amounts of bovine serum albumin [17]. The potential for increased protein binding associated with the reported increase in surface roughness due to fluoride, might explain some of the characteristic features of dental fluorosis. Increased incidence of protein binding may inhibit crystal growth during the important maturation phase of crystal thickening, and result in the tooth erupting with immature (porous) enamel which has retained protein.

The precise nature of protein–crystal binding has yet to be specifically defined, but current opinion favours electrostatic interaction as the principle driver for matrix–mineral association. Recent studies have identified two distinct binding sites on the $a$- and $c$ faces of synthetic HAP which bind either acidic or basic proteins (Ca and P sites, respectively) [18,19]. This work also showed that the ratio of Ca to P sites for a range of calcium phosphate salts was of the order DCPD > OCP > HAP, was independent of bulk mineral stoichiometry and was consistent with surface zeta-potentials. The ratio of Ca to P sites on specific crystal faces would explain differential effects for proteins in the control of crystal habit. Further work using powdered cattle bone indicated that there was an overall greater number of cationic sites compared with anionic sites based upon saturated adsorption measurements for a range of standard proteins [19], suggesting a net negative charge on the crystal surfaces. However, investigations using individual crystals isolated from developing enamel have shown

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Fig. 2. Growth of developing enamel crystals in the AFM. (a) AFM height image and (inset) 3-D rendered appearance of a single developing enamel crystal used in the growth experiments. (b) In vitro growth in crystal width with time under conditions of pH and supersaturation with respect to hydroxyapatite similar to those found in vivo. (c) Decrease in crystal surface roughness with time for the same experiment as in (b) above. Developing enamel crystals showed a decrease in topographical features concomitant with crystal growth.

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1 DCPD = brushite (CaHPO$_4$·2H$_2$O).
that the surfaces carry a net positive charge under in vivo-like conditions [8•], suggesting that binding of acidic matrix proteins might predominate in vivo. Acidic matrix macromolecules associated with mineral crystals are a common theme throughout biomineralisation [20–22•]. A great deal of work has therefore centred around elucidation of the mechanisms governing their interaction with the mineral phase.

3. Mechanisms of protein–mineral binding

3.1. Interaction via specific surface docking

The requirement for a precise spatial configuration of charged groups on matrix macromolecules to match the appropriate sites in the crystal surface has been proposed as a necessary pre-requisite for protein binding. For example, osteocalcin (found in the extra-cellular matrix of calcified tissues) specifically inhibits HAP growth in the 0001 plane and shares a similar spacing between the carboxyl groups on Gla residues (spaced within a section of alpha helix) as that between Ca$^{2+}$ sites on the HAP 0001 surface (inter-atomic spacing = 0.545 nm) [23••]. Inhibition of crystal growth related to specific surface docking of osteocalcin on the HAP 0001 plane was directly observed using AFM. The role of the α-helix as a mechanism for aligning acidic side chains for stereo-specific lattice docking was also shown in studies on the salivary protein, statherin, a known inhibitor of HAP growth [24••]. The acidic pentapeptide sequence situated within a helical domain in the statherin N-terminal, was shown to be strongly bound to HAP while the remaining middle and C-terminal regions of the protein were mobile and interacted weakly. Molecular modelling has been used to understand the interaction of dentine phosphoprotein (DPP) with both OCP and HAP, based upon the stereo-chemistry of its acidic side-chains and the known crystal structures [25•]. Oligopeptides containing PSer–Asp repeats based upon the primary sequence of DPP, were found to match surface sites on both OCP and HAP in an extended conformation, though a β-sheet conformation was specific to the 001 crystal face of HAP alone. Further work using native DPP and synthetic HAP coupled with NMR revealed that the secondary structure of the protein on the crystal surface was indeed indicative of a β-sheet conformation [26•]. This work also showed that complete coverage of the crystal surface would be achieved with very few DPP molecules. Molecular modelling using PSer–Glu peptides and different HAP surfaces suggested that a PSer–PSer–PSer–Glu motif would preferentially bind to 100 and 010 planes, principally driven through specific electrostatic interactions with lattice sites [27••]. Again, the most stable configurations were predicted to comprise a β-strand or α-helix structure, which permitted maximum docking with Ca$^{2+}$ sites in the surface of the lattice.

Predictions based upon molecular modelling are useful adjuncts in interpreting the behaviour of native proteins at mineral surfaces, but it is important to remember that charged groups immobilised at surfaces may behave very differently from the same groups free in solution [28•••]. The phosphate group, for example, which is a potentially important site for the electrostatic interaction of crystal sites with basic groups on proteins, was found to experience a shift in pK$_a$ values of the order of 2 pH units, when immobilised within a monolayer surface [29]. The dissociation behaviour of the same group in the surface of HAP crystals is as yet undefined, though work is currently ongoing to define this.

Evidence for direct docking of protein groups with sites within the crystal lattice is scarce, but recent data describing the inhibition of HAP crystal growth by phosphoserine suggested that the adsorbed amino acid was located within the inner Helmholtz plane of the electrical double layer at the HAP/electrolyte interface [30•]. Electrostatic binding was suggested to occur at neutral pH via the protonated amino group of the phosphoserine with negative charged sites on the HAP surface (either phosphate and/or hydroxyl groups), with the phosphate and carboxyl groups on the phosphoserine orientating away from the surface and each other due to electrical repulsion.

3.2. Electrostatic interactions through participating domains

Not all matrix macromolecules are globular proteins with well characterised secondary structure. Indeed, the principle protein of the developing enamel matrix, amelogenin, forms self-assembled aggregate structures (‘nanospheres’) under physiological conditions and has no apparent secondary structure [31,32••,33,34•]. It is difficult to envisage sterically restricted binding to individual lattice sites in these circumstances, raising the possibility that less-specific interactions based on domain interactions may predominate, perhaps based upon charge density as described for the interaction of osteopontin and streptococcal Protein G with crystals of calcium oxalate [35,36•]. The relative size of amelogenin nanospheres compared with enamel crystals themselves suggests interactions with macro properties—such as overall charge density—rather than with specific recognition sites within the crystal lattice [10••,37••]. In the last year, chemical force coupled with lateral force microscopy was used to generate the first evidence for the existence of surface charge domains on biological
crystal surfaces [10••]. The surfaces of individual developing enamel crystals were shown to comprise of a series of discrete and alternating domains of positive and less positive (perhaps even negative) charge density aligned along and perpendicular to the crystal c-axis. The underlying chemical basis for these domains is not known (given that they do not map directly onto known HAP lattice parameters), but they may represent a chemical ‘memory’ of the crystal origin. Recent studies have suggested that enamel crystals may initially form via a fusion of sub-unit structures into extended linear arrays and that c-axis elongation occurs via addition of matrix/mineral sub-units rather than single ion accretion [34••]. The domains described using CFM may represent the fusion boundaries of these sub-units. Alternatively, it is possible that precursor mineral phases such as OCP, may be detectable contemporaneously with HAP within the crystal and that the boundaries reflect transition planes. It is also possible that the domains are relevant to the Stern layer only and do not reflect underlying lattice chemistry. However, this is the surface with which protein interactions take place, raising the possibility for binding between larger domains (rather than stereo-specific groups) on both crystal surfaces and proteins themselves. Serum albumin, amelogenin nanospheres, DPP and dentinal sialoprotein (DSP) have all been observed to bind to these positively charged domains under conditions of physiological pH [38••,39].

All of these proteins would be expected to carry overall negative charge at physiological pH and, taken together with the CFM data describing the positively charged domains on the crystal surfaces, this points to a net electrostatic interaction (Fig. 3). Selective buffer desorption indicated that many of these proteins were adsorbed to mineral surfaces through such electrostatic interactions [10••,38••,39]. In addition, similar studies indicated that amelogenin was selectively adsorbed to specific crystal surfaces, presenting a possible mechanism for the control of crystal morphology [40]. Supportive evidence for the ability of amelogenin to control crystal morphology comes from studies using OCP. These have shown that amelogenin interacts preferentially with the 010 face of OCP resulting in elongation and thickening of the crystals formed in a model system [37••,40,41]. The resulting OCP crystals had decreased width/thickness ratio when formed in the presence of amelogenin compared with controls, with growth inhibition being greatest in the b-axis > c-axis > a-axis. Similar observations were made when HAP crystals were allowed to grow on Bioglass™ surfaces in the presence of amelogenins, which again resulted in a change of crystal habit towards long, thin crystals with a greater aspect ratio [42].

3.3. Non-electrostatic matrix–mineral interactions

Electrostatic interactions are not the only possible mechanisms underlying matrix–mineral association. Other interactions may also take place, such as hydrogen bonding between protonated groups in the protein and electronegative sites/domains at the crystal surface [38••] and van der Waals forces [27••]. In addition, loss of protein conformation on binding leading to decreased entropy may also favour adsorption. For example, catalase enzyme was shown to bind to synthetic HAP under conditions where both protein and mineral were negatively charged [43]. This was associated with a conformational change in the enzyme. Hydrophobicity is a potentially important driver, particularly in the case of amelogenin, which is highly hydrophobic. Adsorption onto the crystal surface may be thermodynamically more favourable compared with the surrounding aqueous milieu. Recent work using amelogenin in the OCP model described above, has suggested a hydrophobic interaction between amelogenin and the 010 face of OCP, which has no exposed structural water and contains a negatively charged convex region [39]. Investigations to determine the surface tension components for a range of calcium phosphate salts found a wide variation in their relative surface hydrophilicity/hydrophobicity [44••]. Interestingly, the surface of fluorapatite was found to be very hydrophobic compared with DCPD, OCP and HAP, which might increase binding of hydrophobic proteins (such as amelogenin), with important implications with respect to retention of proteins and inhibition of crystal growth in fluorosis.

4. Conclusions

The physico-chemical characteristics of crystal surfaces are important determinants in the binding of matrix macromolecules associated with the control of crystal growth in the mammalian skeleton. Identification of putative growth sites on natural crystal surfaces may be related to growth modalities and key control points in the inhibition of crystal growth. Increased surface roughness increases protein binding and may be an important factor in fluorosis. Protein–mineral binding may be effected via docking of stereo-specific charged residues on matrix proteins with crystal lattice sites providing an instructional template or complementary configuration. Protein secondary structure is a potentially important determinant in maintaining charged side chains in the correct spatial alignment for binding. More general electrostatic interactions between participating domains on matrix proteins and crystal surfaces may preside in vivo. Developing enamel crystal surfaces
Fig. 3. (a) Schematic illustrating use of modified AFM tips to generate friction images of developing enamel crystal surfaces. Electrostatic interactions between the AFM tip and charged groups at the crystal surface result in torsional movement of the cantilever which is translated into a friction image. (b) Areas of high and low friction, corresponding to domains of net positive and less positive or negative charge density were independent of topography and arranged along the crystal c-axis [10]. Matrix proteins, including serum albumin (c) and amelogenin nanospheres (d) were found to bind to the crystal surfaces with similar periodicity under physiological conditions (arrows) [10, 38].

comprise of a series of discrete and alternating domains of a positive and less positive (or even negative) charge density, which have been shown to directly bind a number of matrix proteins under physiological conditions of pH and ionic strength. Hydrophobic interactions may also be an important driver, particularly in respect to selective binding to specific crystal faces which would result in the control of crystal habit. Despite these advances in understanding the role played by crystal surfaces in protein binding, there are still few studies which have used natural crystals from the skeletal tissues or experimental conditions which simulate the in vivo milieu. Future studies need to focus on development of methodologies to permit such investigations. New methodologies for the interrogation of individual crystal surfaces at the molecular level—such as the use of single-walled carbon nanotubes in the atomic force microscopy work—would also increase our understanding of the underlying reasons for the observed pattern of domains. Determination of the dissociation behaviour of charged groups on biological surfaces—both mineral
and protein—would assist in interpretation of mechanisms of binding. Finally, more structural information is required for matrix proteins in solution and bound to crystal surfaces in order to increase our understanding of the interactions involved. Such information will be key to developing biomimetic materials and novel therapeutics.

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References and recommended reading

- of special interest
- of outstanding interest


Comprehensive overview describing the role of enamel matrix proteins in the biomineralisation process, including considerations of specific roles in the inhibition of crystal growth through matrix-mineral binding.


First report describing the use of atomic force microscopy to image the surface characteristics of crystals from skeletal mineral. Changes in surface roughness with crystal maturation discussed in relation to growth mechanisms and matrix binding.


First report describing surface charge characterisation on individual developing enamel crystals by chemical force microscopy. Evidence presented that amelogenin binds to positively charged domains according to a spatially predetermined patterning of the crystal surface.


First report describing growth characteristics of individual crystals of (synthetic) hydroxyapatite in real time using atomic force microscopy. Data revealed growth of 0001 face via multiple 2-D nucleation.


Comprehensive investigation describing the mechanisms of (synthetic) hydroxyapatite crystal growth, detailing the differences between spiral and multinuclear growth.


Effect of carbonate substitution on the kinetics of hydroxyapatite crystal growth. Evidence presented to show that changes in crystal habit arise with increasing carbonate substitution, which has important implications when considering crystals of bone and dentine, which are highly substituted with carbonate and are plate-like rather than needle-like in morphology.


Evidence supporting view that increased crystal surface topography can increase protein binding.


Excellent overview of the role of acidic macromolecules in the biomineralisation process.

Direct observations using atomic force microscopy of inhibition of (synthetic) hydroxyapatite crystal growth on the 0001 plane via stereo-specific surface docking of osteocalcin, a protein found in a number of calcified tissues. Excellent comprehensive study, which also describes the role of secondary structure in maintenance of stereo-specificity.


Comprehensive characterisation of binding of statherin (a salivary protein) to hydroxyapatite surfaces using solid-state NMR. Evidence presented for role of an α-helical conformation in maintaining correct stereo-specific configuration of charged residues in the N-terminal domain for optimum mineral binding.


Molecular modeling of dentine phosphoprotein mineral binding motifs on hydroxyapatite with comprehensive explanation of constraints and characterisation of model. Optimum conformers for binding to hydroxyapatite 001 face predicted to have β-sheet structure for specific docking to crystal sites.


Application of NMR technology to determine conformation of native dentine phosphorin (implicated as an important mediator in the biomineralisation of dentine) bound to (synthetic) hydroxyapatite surfaces. Phosphon polypeptides are shown to be in a β-sheet conformation when bound. Taken together with [26,*], provides a comprehensive characterisation of mineral binding by an important protein matrix.


Motifs based upon the N-terminal domain of the salivary protein statherin used in a molecular modeling of specific protein–hydroxyapatite interactions. Data suggest that preferred faces for binding are governed by electrostatic interactions between the crystal surfaces and peptide. Emphasises on the importance of secondary structure—particularly β-sheet and α-helical conformations in maintaining correct stereo-specific configuration for lattice docking.


Review of effect of surface immobilisation on dissociation of ionisable groups with possible roles in the biomineralisation process. Ionisation shown to be dependent upon ionic strength and to differ by up to 3 pI units from values obtained for the same groups free in solution. These data have important implications with respect to predicting the behaviour of groups at surfaces and therefore protein–mineral interactions.


Evidence for direct docking of phosphoserine within inner Helmholtz plane of (synthetic) hydroxyapatite surface through electrostatic interaction.


Measurements of crystal surface hydrophilicity/hydrophobicity for a range of calcium phosphates revealed that fluoroapatite is relatively hydrophobic compared with hydroxyapatite. This could be an important finding when related to protein binding in fluorosis, where arrested crystal growth occurs possibly due to increased protein binding.