Growth of Calcium Phosphate on Demineralized Dentine Seed Material

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The process of crystal growth of calcium phosphate from supersaturated calcifying solution was initiated by inoculation of demineralized human dentine. As shown by characterization of growth products, various metastable phases of calcium phosphate undergoing continuous qualitative change during the controlled kinetic process are formed, as required by solubility rules.

The process of crystal growth of calcium phosphates has drawn considerable scientific interest, as its proper understanding applies to the many important processes taking place in the nature, more specifically in human environment and living organisms.

It has been shown that crystal growth of calcium phosphate phases can be reproducibly studied by the inoculation of metastable supersaturated solutions of calcium phosphate with seed crystals. This contribution illustrates, that the process of crystal growth of calcium phosphate can also be initiated with demineralized human dentine. The phase changes which occurred during controlled growth process may be of interest for better understanding of processes of biological calcification.

EXPERIMENTAL

The growth of calcium phosphate on the surface of suspended demineralized dentine was performed in a water thermostated double walled vessel. The stable supersaturated calcifying solutions, 0.15 mol dm$^{-3}$ in NaCl, were obtained by mixing calcium chloride and phosphoric acid and by subsequent adjustment of pH = 7.40 with 0.05 mol dm$^{-3}$ carbon dioxide free potassium hydroxide solution. The detailed experimental procedure of crystal growth monitoring was described elsewhere. Total soluble calcium and phosphate were determined by atomic absorption spectrophotometry and by spectrophotometric method, respectively. Seed material was prepared from freshly extracted caries-free teeth and the ground dentine fraction was demineralized by action of EDTA. After demineralization had been completed, the dentine organic matrix contained less than 0.2 ppm of mineral impurities.

The process of crystal growth was controlled by pH-stat (pH = 7.40), and characterized by analytical determination of Ca/P uptake ratio, scanning electron microscopy (SEM) and specific surface area (SSA) determinations of growth product, which were isolated and liophilized at given reaction times. SSA parameter was determined by a dynamic flow method based on the adsorption of dry nitrogen gas from a nitrogen-helium mixture at $-195^\circ$C (»Mono-sorb« surface area analyzer, Quantachrome Cor.).

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RESULTS AND DISCUSSION

The most marked difference between seeded growth with: a) mineral seed and b) organic matrix, is the effect of a very pronounced induction period in the latter case, as demonstrated in Figure 1. The process once started, the kinetic profiles of reaction curves are rather similar, indicating that the growth products undergo a qualitative change, as already demonstrated in the case of hydroxyapatite-seeded growth. This follows from the known thermodynamic solubility parameters of the calcium phosphate phases which may be formed under given experimental conditions. At a very early stage of the growth process several calcium phosphate phases may grow simultaneously, provided that the supersaturation of the solution is sufficient enough to exceed the solubility product for the respective phases. However, as the growth process proceeds, the system becomes undersaturated with respect to some phases. The consequence is the growth of less soluble phases, which proceeds at the expense of the incongruent dissolution of more soluble precursors. The degree of supersaturation with respect to calcium phosphate phases: dicalcium phosphate dihydrate [CaHPO₄ • 2H₂O, DCPD], octacalcium phosphate [Ca₈H₆(PO₄)₄, OCP], tricalcium phosphate [Ca₃(PO₄)₂, TCP] and hydroxyapatite [Ca₅(PO₄)₃OH, HAP] can be estimated at any reaction time by using the expression:

$$S_{CaP} = \frac{iP - K_{sp}}{K_{sp}}$$
where $IP$ represents the appropriate ionic concentration product, $K_{sp}$ the solubility product value for a particular calcium phosphate phase and $S_{CaP}$ the supersaturation ratio. For $S_{CaP} < 0$ the system is undersaturated for a particular phase and dissolution takes place. On the basis of analytical data and thermodynamic solubility product values: $K_{sp}$ (DCPD) $= 2.1 \times 10^{-7}$, $K_{sp}$ (OCP) $= 1.25 \times 10^{-47.5}$, $K_{sp}$ (TCP) $= 1.15 \times 10^{-29.6}$ and $K_{sp}$ (HAP) $= 1.8 \times 10^{-38.7}$ it is shown in Figure 1 that the growth process proceeds through the levels of undersaturation of the phases mentioned, but remains supersaturated with respect to HAP at all reaction times. The consequence should be the ultimate formation of the least soluble phase, HAP, the most important mineral constituent of bones and teeth.

The presence of the sulphonated mucopolysaccharide, chondroitin sulphate, causes a marked inhibition of the mineralization of demineralized dentine. At $10^{-5}$ M level of mucopolysaccharide the growth process was delayed for 15 hours (Figure 1); $10^{-3}$ M level inhibited the growth process for 48 hours. The influence of the mucopolysaccharide described is interesting, as it was suggested that the presence or absence of this class of compounds might be a regulatory mechanism in calcification or bone formation.

An increase in supersaturation and in the reaction temperature leads to a decrease in the induction period. Once started, the process follows several stages, which could be briefly summarized as follows:

1. After the induction period a slow mineralization begins. The SEM photomicrograph of the early mineralization step shows mineral deposition along the cleavages of dentine tubules (Figure 3B). This indicates that the primary mineralization step takes place on a rough rather than on a smooth surface of the organic matrix.

2. Mineralization continues atop the primarily formed deposit and the contours of the material grown indicate that the smooth surface of tubules may still be free of mineral deposit. The spongy structures formed consist
of platelet crystals (Figure 3C), probably OCP<sup>9</sup>, which is a stable phase under experimental conditions attained, as shown in Figure 1. At the reaction time of 300 min the increasing SSA parameter approaches the maximum value, which indicates the formation of metastable calcium phosphate phases<sup>3</sup>.

3. At later reaction times a characteristic change in the morphology of the mineral material grown was noticed (Figure 3D). The platelet crystal aggregates became covered with a denser phase, which probably accounted for the recorded decrease of the SSA parameter. These changes could be attributed to the parallel processes of OCP dissolution and growth of less soluble HAP, because under these reaction conditions the system became undersaturated with respect to OCP.

4. At 1000 min reaction time the minimum in SSA parameter was recorded; at this stage of the growth process the clear calcifying solution became turbid, probably due to the fluid shear of large agglomerates. The formation of the »secondary« phase accounts for a new increase in the SSA parameter. As shown in Figure 3E, further changes in surface morphology clearly demonstrates surface transformation and HAP formation.

Figure 3F shows a histological cross section of mineralized dentine, isolated after 1000 min of growth. It reveals that under the experimental conditions used the mineralization process takes place only on the exposed surface of dentine, but not inside the body of the organic matrix.

The constancy of the uptake reaction ratio, ΔCa/ΔP = 1.45 ± 0.03 is well documented in Figures 1 and 2. This ratio is too low to demonstrate exclusive formation of HAP, which may be a slow, surface controlled process if proceeding via the dissolution of OCP.

When the growth process was prolonged for 4 days the analysis of mineral deposit showed a Ca/P ratio of 1.57. This material contained approximately 6 molar % of sodium, which is in good agreement, with Newman's statement that sodium can replace calcium in the apatitic lattice<sup>10</sup>.

The results reported clearly indicate that the demineralized dentine may serve as a suitable seed material for the growth of the mineral component of bone mineral. The process of mineralization described could be taken as a simplified model of the process taking place in vivo.

REFERENCES

Figure 3. Scanning electron micrographs of mineralized dentine, isolated at reaction times:
0 (A), 130 min (B), 300 min (C), 600 min (D), 1000 min (E) and 1000 min (F).

$t = 37^\circ C$, 0.15 M NaCl, initial Ca/P solution ratio 1.66.
SAŽETAK
Nastajanje kalcijevog fosfata na demineraliziranom dentinu

B. Tomazic

Proces kristalnog rasta kalcijeva fosfata iz prezasićene stabilne otopine kalcijeva fosfata započeo je cijepljenjem s demineraliziranim dentinom humanog podrijetla.

Karakterizacijom taloznih produkata, koji nastaju u toku kontroliranog kinetičkog procesa, pokazuje se stvaranje različitih metastabilnih produkata, podložnih kontinuiranoj kvalitativnoj promjeni, u skladu sa zakonima topljivosti.

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