A MOLECULAR MECHANISM FOR BIOLOGICAL CALCIFICATION

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SUMMARY

A general mechanism for calcification in biological systems is outlined which consists of organization of macromolecules and simultaneous or subsequent binding of alkali cations (principally Ca^{2+}) by the molecules to create compartments with higher pH values than the bulk phase of the solution. The higher pH value in this microcompartment would raise the concentration of PO_4^{3-} and OH^- to an extent sufficient to induce their precipitation with Ca^{2+}.

Calcification in biological tissues occurs on a variety of matrix materials. Collagen is the major matrix in bone and dentin (1); non-collagen proteins are the matrix for dental enamel (2); while in aorta, elastin appears to be the material inducing calcification (3). Although mucoproteins, mucopolysaccharides, and lipids also have been found in association with these matrix proteins, no clear explanation exists for the molecular mechanism by which any of these macromolecules promotes nucleation of hydroxyapatite. Since purified collagen (1,4) or isolated elastin (3) in simple, buffer solutions will initiate nucleation, active cellular processes are not an obligatory requirement. A general mechanism for macromolecules containing certain functional groups and spacial relationships could explain the process. This paper describes such a mechanism which would have general applicability to systems containing a macromolecule in an aqueous environment. In brief, it is that macromolecules can create alkaline microcompartments which increase the concentration of tribasic phosphate and hydroxyl ions.

Hydroxyapatite consists solely of calcium, tribasic phosphate and hydroxyl ions arranged as a right, rhombic prism in a unit cell with a length along each edge of the basal plane of about 9 Å and a height of about 6 Å (5).

Since the anions in this precipitate are those whose concentrations are readily increased simply by raising the pH of a solution containing phosphate ions, it is apparent that the pH at the site of precipitation can control both nucleation and growth of the crystal.

Hartley and Roe (6) demonstrated that the pH value on the surface of a colloid could differ by as much as two pH units from the pH value in the bulk of the suspending solution. The activity of trypsin embedded in polyelectrolyte gels differs in a manner predicted from the effect of the electrostatic charge of the gel on the pH value in the microenvironment of the enzyme (7). A pH gradient of two or more units between the microenvironment of the gel and the bulk phase of the solution was calculable.

Addition of Ca^{2+} or alkali metal cations to isolated mitochondria or mitochondrial subfractions is characterized by stoichiometric binding of the cation to the biological material and release of protons to the suspending solution (8). This appears, in part at least, to be independent of respiration (9,10) and may be merely a translocation of protons from a mitochondrial protein to the suspending solution which is produced by the binding of Ca^{2+} to the protein. A similar translocation of protons from Ca^{2+} binding was observed for the muscle protein, troponin (11).

Many investigators (12-18) consider the binding of calcium to a macromolecule as the first and most important event in the process of calcification. It has been proposed further (19,20) that this calcium is bound to sulfate and carboxyl groups. Many of these investigators found clear experimental evidence for calcium binding as an absolute requirement for initiating calcification.

The only questions remaining before application of this concept to calcification in the vicinity of the macromolecular binding site are the magnitude of the pH rise in the vicinity of the bound Ca^{2+} and the volume of the microcompartment throughout which this elevated pH value exists. For their effect on inducing precipitation of hydroxyapatite, the two

quantities, pH rise and compartmental volume, must be considered simultaneously. So long as the requirement is met for a sufficient pH rise to produce the concentrations of PO_4^{3-} and OH^- which exceed their solubility product constant with Ca^{2+} in a compartment which is large enough for the crystal to form, then nucleation will occur. Maintaining this alkaline pH and supply of reactants on the surface of the unit crystal will allow the crystal to grow.

The macromolecules inducing calcification in bovine dental enamel can be used to formulate an example. These matrix proteins have been characterized by X-ray diffraction (21). The molecules are arranged in parallel arrays with approximately 10 Å separation between the pleated sheets. Approximately every fourth amino acid is a proline residue; a 180 degree bend in the polypeptide chain may occur at each proline residue. Furthermore, the proteins are acidic (22) which means that they contain a net surplus of anionic or other proton accepting groups. These are the same types of groups which have a high affinity for Ca^{2+} (23) and from which Ca^{2+} can displace protons (24).

Figure 1 shows the approximate spacial relationship proposed for the matrix protein molecules from bovine dental enamel, the Ca^{2+} bound to anionic residues, the alkaline compartment created by the bound Ca^{2+} (represented by OH^- adjacent to the bound Ca^{2+}), and the hydroxyapatite crystals. The 10 Å space between the polypeptide sheets should be sufficient for the nucleation of a unit crystal with a 9 Å edge. Crystal growth would be accompanied by outward displacement of the alkaline, calcification-inducing sheet.

Although the extent of the reduction in the chemical potential of the proton in the interior of the protein compartment is not known at this time, it is easily conceivable that such a reduction equivalent to one pH unit gradient between the interior and the bulk of the solution could exist. Such a pH gradient, from pH 7 to pH 8, would increase the PO_4^{3-} concentration 15 fold. This would greatly increase the ion product for hydroxyapatite.

Approximate spacial representation for calcification within enamel protein.

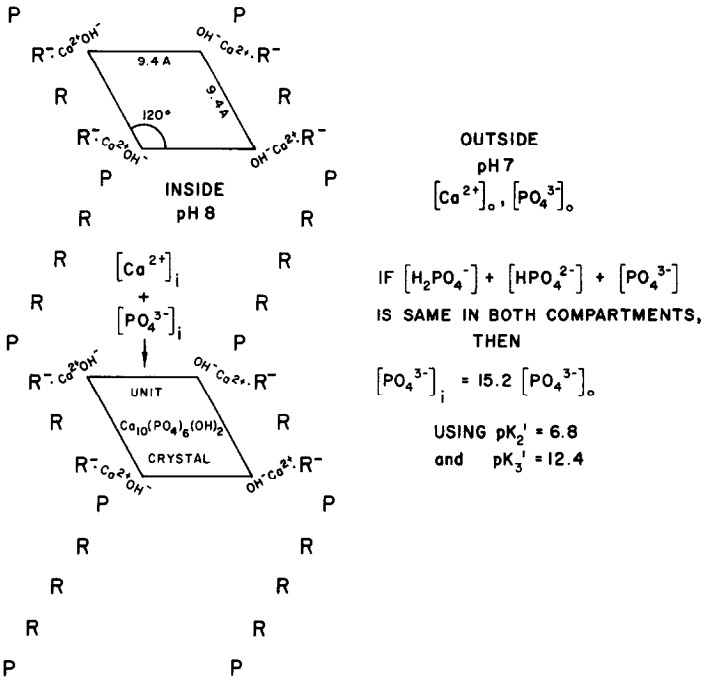

Fig. 1: The alkaline microcompartment is created by Ca^{2+} bound to amino acid residues and contains unit hydroxyapatite crystals. $[Ca^{2+}]o$, $[PO_4^{3-}]o$, $[Ca^{2+}]i$, and $[PO_4^{3-}]i$ represent the concentrations of these ions in the outside and inside compartments, respectively. P represents a proline residue and R represents unidentified amino acids.

In summary, the general mechanism for calcification would be organization of macromolecules and simultaneous or subsequent binding of alkali cations (principally Ca^{2+}) by the molecules to create compartments of higher pH than the bulk phase of the solution. The higher pH in this microcompartment would raise the concentration of PO_4^{3-} and OH^- to an extent sufficient to induce their precipitation with Ca^{2+}.

It is readily apparent how other ions which interfere with calcification could be binding to these same sites. Molecules such as tetracycline, etc. could be binding directly to these anionic sites or to the bound calcium; the latter possibly would be more consistent with recent data (25,26). These

molecules or ions thus could exert their inhibiting effect either by enlarging the compartment or decreasing the alkaline pH. Either of these could reduce the critical elevation of pH throughout the minimal volume necessary for nucleation of a unit crystal.

Höhling et al. (27) found 6-8 sites for nucleation in the "hole zones" of collagen in dentin and bone and about half as many sites in the "overlapping zones." Within the relatively large "hole zones", several alkaline areas could exist to favor precipitation of hydroxyapatite. This could account for the lower degree of uniformity in the final hydroxyapatite in dentin and bone compared to that in enamel.

Although this mechanism has been formulated for hydroxyapatite, the same principle could apply to precipitation of calcium carbonate. However, with $CaCO_3$ some additional mechanism must prevail which regulates the relative concentrations of phosphate and bicarbonate in the external or bulk phase. For example, an increase in the concentration of PO_4^{3-} due to a rise in pH from pH 7.4 to pH 8.4 would have more than 100 times greater effect on the ion product for $Ca_3(PO_4)_2$ than on that for $CaHPO_4$ in normal human extracellular fluid and 10 times greater on the ion product for $CaCO_3$ than on that for $CaHPO_4$.

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