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The effect of Leucine on the crystal growth of calcium phosphate

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Abstract Crystallization kinetics of hydroxyapatite, HAP, in the presence of Leucine, a natural aminoacid with hydrophobic side groups, were investigated at conditions of sustained supersaturation, 37 °C, pH 7.40, ionic strength 0.15 M. In the presence of Leucine, the crystal growth rates of HAP decreased markedly. This action is due to adsorption and subsequent blocking of the active growth sites onto the surface of the HAP crystals. The kinetic results revealed that a Langmuir-type adsorption isotherm is followed and an affinity constants of $k_{aff} = 20.26 \times 10^2$ L/mol for HAP crystal surface were calculated. The apparent order of the crystal growth reaction was found to be equal to two, suggesting a surface diffusion controlled mechanism.

Introduction

Leucine is the most common amino acid found in proteins, and is essential for optimal growth in infancy and childhood and for nitrogen equilibrium in adults. It is suspected that Leucine plays a part in maintaining muscles by equalizing synthesis and breakdown of proteins [1–3]. The general structure formula of Leucine is shown in Fig. 1. The amine group, $-NH_2$, of the organic side chain categorizes the amino acid as basic with similar pka values as the aminoacid alamine [4]. Also, it has been proved that it enter into the cell environment by simple diffusion [5, 6]. It is obvious that studies of such molecules of biological relevance onto calcium phosphate growth can be related to important processes of desirable or pathological calcification [7–10].

Among calcium phosphate phases, hydroxyapatite [HAP, $Ca_5(PO_4)_3(OH)$] is thermodynamically the most stable calcium phosphate salt and is the inorganic component of hard tissues such as bones and teeth [4, 7]. On the other hand, calcification is the most frequent cause of the clinical failure of cardiac valve bioprostheses [11, 12]. The mineral deposits of the human atherosclerotic aorta consisted mainly of calcium apatite (71%), carbonate (9%) and contained high percentage of protein (15%) [13, 14]. The average Ca/P ratio was ~1.7 in mature atherosclerotic plaque biomineral and mature skeletal biomineral, both of which approximate HAP in composition. Treatment with amino acids appears to prevent the calcification of cardiac bioprostheses [15].

In the present work, the effect of Leucine on the HAP crystal growth was studied by the constant composition method [16–18]. This technique is advantageous compared to other methods used for studying nucleation, crystallization and dissolution of salts, mainly because the concentrations of the solution species remain constant during the course of the experiment.

Experimental procedure

Crystal growth experiments were conducted in a 250 mL, thermo stated at 37 °C, double-walled water-jacketed glass cell, connected to a recirculating water bath, by using the

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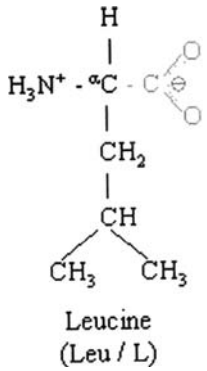


Chemical formula	$C_6H_{13}NO_2$
Molecular mass	131.18 g mol^{-1}
Melting point	293 °C
Density	1.165 g cm^{-3}
Isoelectric point	5.98
pK_a	2.32 9.58

Fig. 1 The structural formula and chemical properties of the aminoacid Leucine

constant composition technique. Continuous monitoring of pH was performed using a combined glass/Ag/AgCl electrode (Metrohm, 6.0202.100), equilibrated at 37 °C, standardized before and after each experiment with NBS standard buffer solutions [19]. The reaction kinetics was observed with an appropriately modified pH-stat system (Radiometer pH-meter 26, Radiometer titrator 11, Autoburette ABU1C). A pH change as much as 0.005 pH units, which followed HAP precipitation, triggered the addition of titrants from two mechanically coupled burettes of the controlling system. The solution contents of two burettes were as follows: the first one had $CaCl_2$ and NaCl, while the other KH_2PO_4 and KOH, at appropriate concentrations so that no change in the supersaturation level or the desired stoichiometry in the working solution occurred [20]. As a result the HAP crystallization took place under conditions of constancy of the supersaturation and the concentration of the reactants. The solution pH was adjusted to 7.40 by the slow addition of standard potassium hydroxide solution (Merck, Titrisol), ionic strength was 0.15 M in NaCl and the temperature was adjusted at 37 ± 0.1 °C.

Experimental conditions were chosen in order to physic chemically resemble the biological ones (i.e., the ionic strength was 0.15 M and the pH was equal to 7.40 both similar to that of the human blood).

Special care was taken in order to give sufficient time to the solute Leucine to interact with the HAP crystal surface into the working solution environment. This was achieved by letting phosphates, HAP seed crystals and the Leucine amino acid tested at the desired pH and ionic strength in co-existence prior the introduction of the calcium ions, which initiated the crystallization process. Following the addition of calcium ions, we delay the start of the experiment for 2–4 s until the pH value become to the primary adjustment value of 7.4. Blank experiments with and without the preequilibration procedure gave the same initial rates within the experimental error. This is considered as an improvement compared to classical constant composition method, because adsorption of the solute does not happen simultaneously with the crystal growth of the seed crystals. Thus the phenomena mentioned above can be evaluated and appreciated separately. As a result the reproducibility of the measured crystal growth rates of HAP was better that 2%.

A magnetic rod-like stirring bar continuously stirred working solutions. High purity gas nitrogen, presaturated with water, was bubbled through the solutions before and during the course of the experiment in order to exclude diluted carbon dioxide.

Constancy of the calcium and phosphate reactants during the experiments (to within ±3%) was verified by periodically withdrawn aliquots from the working solution. The samples taken were filtered through membrane filters (Millipore 0.22 μm) and analyzed as described elsewhere [21]. The solids removed by filtration were examined by scanning electron microscopy (Jeol GSM 5200 and LEO SUPRA 35VP), X-ray powder diffraction (Philips PW 1830/1840 using Cukα radiation Ni filter), infrared spectroscopy (KBr pellet method, FT-IR Perkin Elmer 16-PC), specific surface area (multiple point B.E.T. method Perkin Elmer Sorptometer 212 D) and chemical analysis [17] (which gave a molar ratio of calcium to phosphates of 1.67 ± 0.02).

In all experiments stock solutions of calcium dichloride, potassium dihydrogen phosphate and sodium chloride were prepared from the respective crystalline solids (reagent grade, Merck) using triply distilled CO_2-free water. Their standardization has been described in details elsewhere [17, 18, 22].

The amino acid Leucine, were purchased from Sigma Chemicals (extra pure more than 99%, L-chiral configuration). Stereochemistry of amino acids is of critical importance because it is related to their adsorption behavior. Thus only the L-form, which is present in physiological systems, was used in the crystallization experiments (at the experimental conditions applied did not happen racemization [6].

Results and discussion

Leucine had no significant buffer activity in the physiological pH range where all crystal growth experiments were done [6]. In the concentration range where all kinetic experiments were done the amino acids was diluted [5].

Concentrations of all ionic species in the working solution during the crystallization process were calculated from dissociation constants for phosphoric acid and the equilibrium constants for the ion-pair formation involving calcium, potassium and sodium, which are present in the solution, with the phosphate anions. Dissociation constants and ion-pair formation of the amino acid were also taken

under consideration [23, 24]. The stability constants of the amino acids used were modified to 37 °C using the Van't Hoff equation (values at 25 °C).

Experiments with different amounts of seed crystals (10, 15, 20 and 40 mg) showed the same initial crystal growth rates normalized per unit surface area of the crystal substrate indicating that the crystallization process happens without being accompanied by either spontaneous or secondary precipitation. Experiments with larger amounts of seed crystals were not performed because at ionic strength of 0.15 M in NaCl this could lead to aggregation of the solid particles. Changes in the stirring rate between 100 and 750 rpm had no effect on the growth rate suggesting that the rate determining step of the process is not bulk diffusion from the bulk solution to the crystal surface [25].

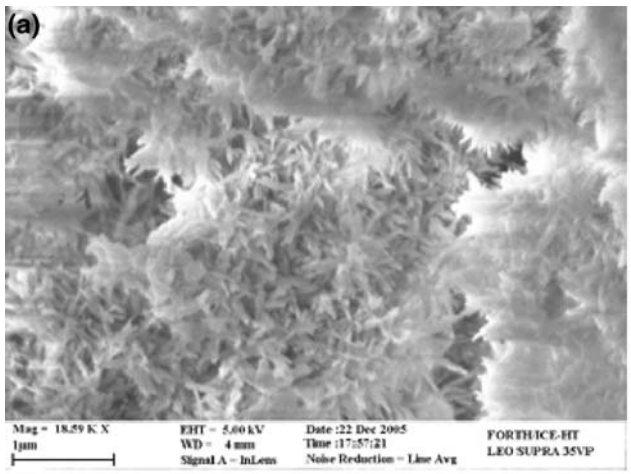
The driving force ΔG for the crystallization process may be expressed as the free energy of transfer from supersaturated solution to equilibrium.

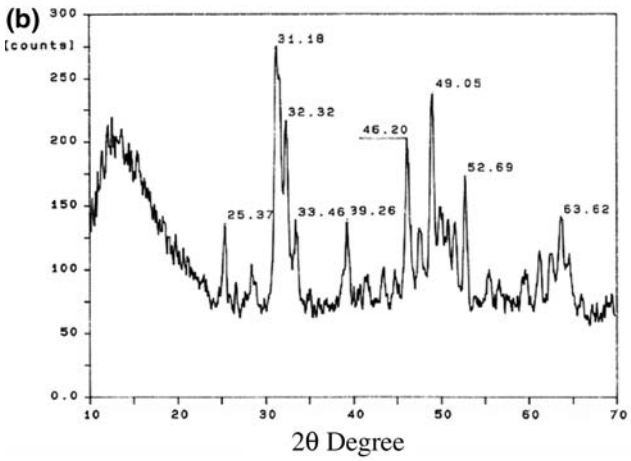
$$\Delta G = -\frac{R_g T}{\upsilon} \ln \frac{IP}{K_s^o} = -\frac{R_g T}{\upsilon} \ln \Omega \tag{1}$$

In Eq. 1, IP is the ionic product of the precipitating salt, K_s^o its thermodynamic solubility product at 37 °C (2.35×10^{-59} for HAP), υ the number of ions in a unit formula of the precipitating calcium phosphate phase (e.g. 9 for HAP), R_g the gas constant, T the absolute temperature and Ω the supersaturation ratio.

The ionic product of the supersaturated solution was calculated taking into account all equilibriums. The computation of the solution speciation was made using expressions for mass balance, electro neutrality, and ion association equilibrium constants. The procedure involved successive approximations for the ionic strength [16] and for this reason a computer programmed software was developed [26].

In the presence of the Leucine tested the Gibbs free energy is not changing appreciably (as concluded from the solution speciation) even at the highest concentration levels used. This point to the fact that changes in the crystal growth rate cannot be attributed to reduction of the solution supersaturation due to ion pair formation of the solute amino acid with one or more of the reactants. The data are corrected for changes in surface area, which is reduced during the crystal growth process [27]. In all cases well crystallized HAP is exclusively formed as was confirmed by Scanning Electron Microscopy (Fig. 2a), X-ray powder diffraction Fig. 2b), and FT-IR spectroscopy of the filtered solids (Fig. 3), which is in accordance with the calculated thermodynamic driving forces ΔG_{HAP} as shown in Table 1. The Ca/P ratio of the solid precipitated was 1.67 ± 0.2 which is in accordance with the Ca/P ratio of the Calcium and Phosphate consumption from the two titrant solutions. Any deviation from this ratio (mentioned in the experimental section and in Table 1) lead to deviation in the constant composition of the working solution which is resulting in large pH changes and lost of the control of the electronic system along with serious changes in total Calcium and Total Phosphate concentrations in the working solution. Crystal growth rates were found to be proportional to the relative solution supersaturation, σ, with respect to HAP, defined as [28]:





2θ	25.37	31.18	32.32	33.46	39.26	46.20	49.05	52.69	63.62
hkl	002	211	300	202	130	222	213	004	304

Fig. 2 (**a**) Scanning electron micrograph of HAP crystallization on HAP seed crystals in the presence of 7.62×10^{-4} mol/L Leucine, (**b**) X-ray diffraction pattern of the above mentioned crystals (ASTM card file No 9-432), along with the *hkl* Miller index

$$\sigma = \frac{(IP)^{1/9} - \left(K_s^o\right)^{1/9}}{\left(K_s^o\right)^{1/9}} = \Omega^{1/9} - 1 \tag{2}$$

$$R = k\, s\, \sigma^n \tag{3}$$

where R, is the overall crystal growth rate, k the rate constant, s a function of the number of the active growth

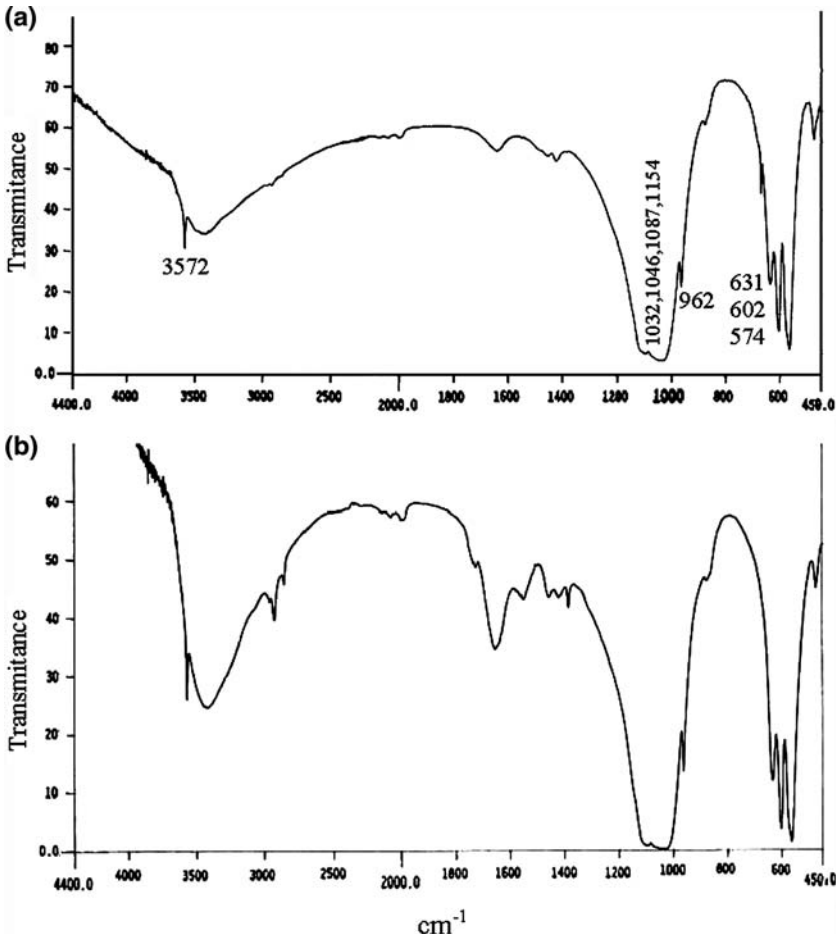


Fig. 3 FT-IR spectra of: (**a**) pure HAP seed crystals [17, 26] and (**b**) HAP precipitation on HAP seed crystals in the presence of 7.62×10^{-4} mol/L Leucine

sites on the crystal surface and *n* the apparent order of the crystallization process. Logarithmic plots according to Eq. 3, yielded straight lines in the presence and absence of Leucine (Fig. 4). From the slope of the linear plots a value of 1.87 ± 0.13 was obtained in the case of the HAP crystallization in the absence of any additive while in the

Table 1 Crystallization of HAP on HAP seed crystals in the presence of Leucine at pH 7.40, 0.15 NaCl, and Calcium (Ca_t): Total Phosphate (P_t) = 1.67

Ca_t (10^{-4} mol/L)	Leucine (10^{-4} mol/L)	ΔG_{HAP} (kJ/mol)	R (10^{-8} mol min^{-1})
5.0	–	–4.43	11.30
4.0	–	–3.94	7.44
3.5	–	–3.63	6.25
3.0	–	–3.29	3.67
2.5	–	–2.88	2.63
5.0	1.91	–4.43	8.23
5.0	3.81	–4.43	7.66
5.0	7.62	–4.43	6.64
5.0	11.43	–4.43	3.88
5.0	15.24	–4.43	2.45
4.0	7.62	–3.94	4.37
3.5	7.62	–3.63	3.65
3.0	7.62	–3.29	2.16
2.5	7.62	–2.88	1.54

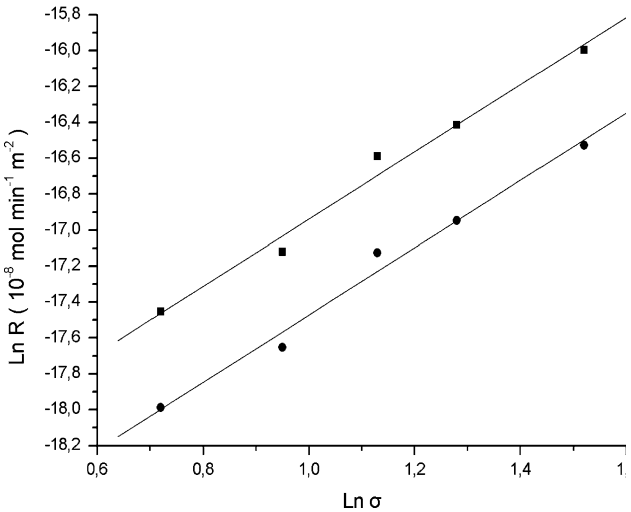


Fig. 4 Kinetics of HAP crystal growth on the presence (•) and in the absence (■) of 7.62×10^{-4} mol/L Leucine according to Eq. 3

presence of Leucine the value for n were 1.88 ± 0.13, respectively suggesting a surface diffusion controlled mechanism [29].

The presence of a foreign compound in the supersaturated solution in which the HAP growth process is taking place, results in the interaction of the solute species with the precipitating solid. When the solute species have functional groups such as carboxyl and/or amino groups, they may adsorb reversibly onto the HAP crystal surface, which contains centers of positively and negatively charged ions. Interactions of Leucine with HAP crystal surfaces lead to adsorption, which follows a Langmuir adsorption isotherm [8, 30, 31]. Thus, the interaction and the kinetic results may be interpreted by the Langmuir formalism [32]. According to this equilibrium model adsorption and desorption of the solute onto the surface are equal:

$$k_{ads.}(1-\theta)\, c_{eq.} = k_{des.}\theta \tag{4}$$

where $k_{ads.}$, and $k_{des.}$ are the specific rate constants for adsorption and desorption respectively, θ the fraction of the crystal surface active growth sites occupied by the adsorbed solute and $c_{eq.}$, the concentration of the additive. The growth rates are depending on the surface coverage θ and so [33]:

$$R_i = R_o\,(1-\theta) \tag{5}$$

where R_i and R_o are growth rates in the presence and absence of the additive, respectively. Combining of Eqs. 4 and 5, gives:

$$\frac{R_o}{R_o - R_i} = 1 + \frac{1}{k_{aff.}}\,\frac{1}{c_{eq.}} \tag{6}$$

In Eq. 6, $K_{aff.}$ is the affinity constant (equal to $k_{ads.}/k_{des.}$) and it is a measure of the affinity of the adsorbate for the adsorbent. As may be seen in Fig. 5, a straight line was obtained, suggesting the validity of the assumed model. The affinity constant, as determined from the slope of the linear plot of $R_o/(R_o–R_i)$ against $1/c_{eq.}$, according to Eq. 6, is $k_{aff} = 20.26 \times 10^2$ L/mol.

The affinity constant is a measure of the affinity of the solute for the surface of the adsorbate and similar values for comparison are given in Table 2. Thus the increased affinity value is related to an increased adsorption that should have a stronger retarding effect on HAP crystal growth rate but geometrical factors must also be taken into account. It is also noteworthy to mention that the Leucine is inhibiting HAP crystal growth even if it is tested at concentration levels of the same order of magnitude with the corresponding value at blood plasma [38]. Therefore, Leucine can be a calcification inhibitor, though a not very effective one as compared to other known inhibitors [39–41]. The advantage of using physiological substances such as Leucine to prevent pathological formation of HAP over the commercial synthetic drugs, which have been accused of being responsible for several side effects, is that the concentration excess of the amino acids in the tissue can be regulated via physiological pathways and therefore the liver function is not charged.

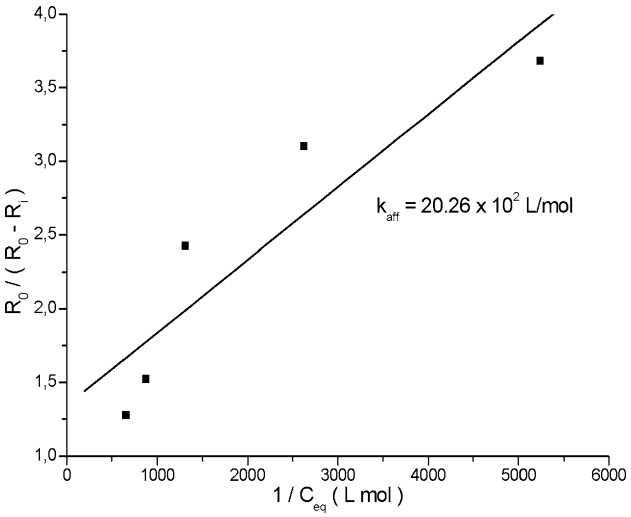


Fig. 5 Kinetics of HAP growth in the presence of various concentrations of Leucine according to the Langmuir kinetic model (Eq. 6); 37 °C, pH 7.40, 0.15 M NaCl

Table 2 Affinity constants for various aminoacid inhibitors of HAP crystal growth

Inhibitor	$K_{aff} \times 10^2$ L/mol	Ref
Alanine	2.86	[4]
Phenylalanine	24.39	[4]
Proline	5.74	[4]
Methionine	6.21	[4]
Lysine	8.77	[34]
Aspartic acid	41.66	[35]
Glutamic acid	30.21	[35]
Serine	9.01	[36]
Tyrosine	30.30	[36]
4-Hydroxyproline	7.46	[36]
Cysteine	6.64	[37]
Cystine	31.41	[37]
Glutamine	34.72	[37]
Glycine	17.14	[37]
Leucine	20.26	This work

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