Interactions of Aspartic Acid, Alanine and Lysine with Hydroxyapatite

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Adsorption of amino acids (aspartic acid, alanine and lysine) on synthetic hydroxyapatite (HAP; $Ca_{10}(PO_4)_6(OH)_2$) was investigated. The surface ion (calcium or phosphate ion) with the same sign of the electric charge as that of the terminal group of the adsorbed amino acid was released from HAP. Aspartic acid and lysine showed high affinity to HAP in weak acidic solution and in weak alkaline solution, respectively, owing to the opposite charges of HAP and these amino acids. On the other hand, the affinity of alanine was low and almost independent of the solution pH. It was concluded that the dominant factor for the adsorption is the electrostatic interaction between the amino acid and the HAP surface. This interaction was shielded by the addition of an indifferent salt for HAP: KCl. However, when calcium or phosphate ion was added to the solution, the adsorbed amount of aspartic acid or lysine increased, respectively, due to the increase in the opposite surface charges to that of the adsorbed amino acid.

Keywords hydroxyapatite; adsorption; amino acid; electrostatic interaction; release; incongruent dissolution; aspartic acid; alanine; lysine

Hydroxyapatite (HAP; $Ca_{10}(PO_4)_6(OH)_2$), which is the most stable phase of calcium phosphates under physiological conditions, is the prototype of hard tissues such as teeth and bones. Regulator proteins for calcification[3] take part in the mineralization process of these hard tissues. Recently, hydroxyapatite column chromatography[4] has come to be used extensively for the separation of proteins. However, the complexity of conformation and diversity of side chains of proteins made the elucidation of their adsorption mechanisms difficult. Studies on adsorption of amino acids on HAP had been performed in connection with the latter problem, *i.e.*, the role of the side chains in the adsorption process. In spite of the simple molecular structure of amino acids, however, some discrepancies exist in the literature. For example, some authors[5,6] explained the adsorption in terms of the Langmuir equation and others[7] did not. In the present paper, the interactions of amino acids with HAP will be discussed in detail, taking into consideration the adsorbed amount of amino acids, the dissolved amount of HAP, and the effects of pH and added salts. Since amino acids can be classified into 3 groups of acidic, neutral and basic, aspartic acid (Asp), alanine (Ala) and lysine (Lys) were chosen as representative amino acids in the present study.

Experimental

Materials HAP was prepared by mixing stoichiometric amounts of CaO and H_3PO_4 in boiling water under a nitrogen atmosphere.[8] The X-ray powder diffraction patterns of the prepared sample were typical of HAP. The Ca/P ratio determined by chemical analyses was 10/6.08, which is almost stoichiometric.The point of zero charge (PZC) of the sample determined by pH titration was 6.4. Sodium L-asparaginate (AspNa) and L-α-alanine (Ala) were purchased from Wako Pure Chemical Industries Ltd. L-Lysine monohydrochloride (LysHCl) was purchased from Nakarai Chemicals Ltd. These amino acids were of reagent grade and were used without further purification.

Methods HAP was suspended in an aqueous solution of an amino acid of known concentration at 30 °C with the mixing ratio of HAP to the solution of 40 g/l. The suspension was shaken continuously. After attainment of adsorption and dissolution equilibrium (incubation was for 24 h), the suspension was filtered through a Sartorius membrane filter (0.2 μm pore size) or a Millipore filter (0.1 μm pore size, this filter was used for the separation of very finely dispersed particles formed in the alkaline media) and the filtrate was used for the chemical analyses.

The concentration of the amino acid was determined by fluorometry using *o*-phthalaldehyde and 2-mercaptoethanol according to the method of Roch.[9] The fluorescence reaction was performed at pH 9.0 (sodium tetraborate–HCl buffer) for Asp and Ala, and at pH 6.5 (sodium dihydrogen phosphate–KOH buffer) for Lys. Emission of the fluorescing reaction product was monitored at 475 nm with excitation at 335 nm using a Jasco FB-550 spectrofluorometer. The concentration of calcium ion (Ca^{2+}) in solution was determined by ethylenediaminetetraacetate chelatometry with 1-(1-hydroxy-2-naphthylazo)-6-nitro-2-naphthol-4-sulfonate (BT indicator) at pH 10. The activity of Ca^{2+} was determined by using an Orion calcium-sensitive electrode (type 93-20) connected to an Orion Microprocessor Ionalyzer (model 901). The concentration of phosphate ion (Pi) in solution was determined by the molybdenum blue method of Gee *et al.*[10] The phosphate ammonium molybdate complex formed was reduced with stannous chloride. The absorbance of the resulting color was measured at 720 nm after 15 min on a Shimadzu model UV-180 spectrophotometer. The pH of the filtrate was measured on a Toa HM-5ES pH meter.

Results

Adsorption of Amino Acids on HAP and Concurrent Release of Ca^{2+} and Pi from HAP at Moderate pH Figure 1A shows the adsorption isotherms of the three amino acids (AA: Asp, Ala or Lys) on HAP from aqueous solutions of AspNa, Ala and LysHCl. The adsorbed amount (X_{AA}: X_{Asp}, X_{Ala} or X_{Lys}) increased with the concentration of the amino acids ($[AA]_f$: $[Asp]_f$, $[Ala]_f$ or $[Lys]_f$), and X_{Asp} is obviously larger and X_{Lys} is somewhat larger than X_{Ala}. Figure 1B shows the equilibrium pH ($(pH)_f$) of the solution, where $(pH)_f$ was almost constant at 6.45.

Figures 2A and B show the equilibrium concentrations of Pi ($[Pi]_f$) and Ca^{2+} ($[Ca^{2+}]_f$), respectively, as a function of the equilibrium concentrations of the amino acids. When HAP was suspended in an aqueous solution of AspNa, $[Pi]_f$ increased and $[Ca^{2+}]_f$ decreased with an increase in $[Asp]_f$. On the other hand, when HAP was suspended in a LysHCl solution, $[Pi]_f$ decreased and $[Ca^{2+}]_f$ increased with $[Lys]_f$. In the case of the HAP–Ala system, pronounced change was not observed in $[Pi]_f$ and $[Ca^{2+}]_f$. In Fig. 2C, the activity of Ca^{2+}, (Ca^{2+}), is plotted against the equilibrium concentrations of the amino acids, where (Ca^{2+}) increased or decreased in the same manner as $[Ca^{2+}]_f$.

Effect of pH on the Adsorption and Dissolution Behaviors In Figs. 3A, B and C, the adsorbed amounts of Asp, Ala and Lys, in the presence (closed symbols) or absence (open symbols) of KCl, are plotted against the equilibrium pH ($(pH)_f$) of the solution. Potassium chloride is an indifferent

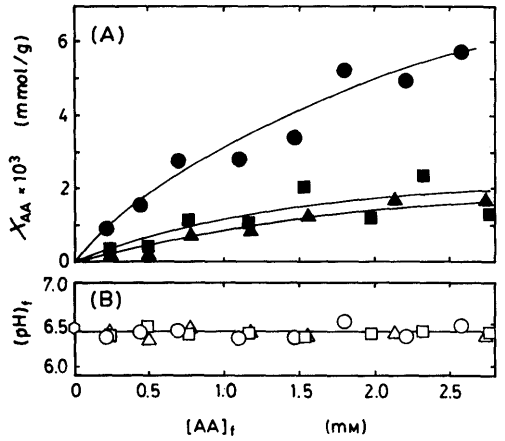

Fig. 1. Adsorbed Amounts of Asp, Ala and Lys on HAP from Aqueous Solutions of AspNa, Ala and LysHCl (A), and Equilibrium pH of Solution (B) as a Function of Equilibrium Concentration of Amino Acid

No buffering agents were used in order to avoid their effects on the properties of the HAP surface and the amino acids. Amino acid: none (○), Asp (●, ○), Ala (▲, △) and Lys (■, □).

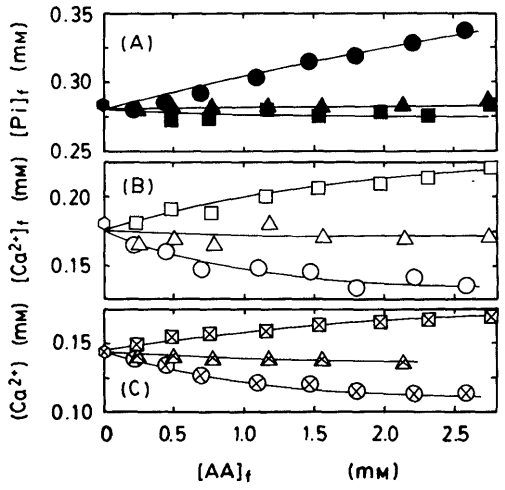

Fig. 2. Relationships between Equilibrium Concentrations of Amino Acids and Those of Pi (A) and Ca^{2+} (B), and Activity of Ca^{2+} (C) in Solution

The experimental conditions are the same as those in Fig. 1. Amino acids: none (●, ○, ⊗), Asp (●, ○, ⊗), Ala (▲, △, ⊗), and Lys (■, □, ⊠).

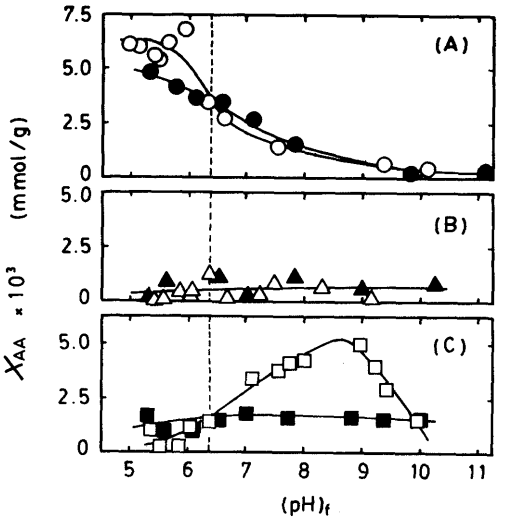

Fig. 3. Relationship between Equilibrium pH of the Solution and Adsorbed Amount of Asp (A), Ala (B) and Lys (C)

Initial concentrations of the amino acids were kept constant at 1.6 mM, and the solution pH was adjusted by addition of HCl or KOH. Amino acids: Asp (○, ●), Ala (△, ▲) and Lys (□, ■). KCl added: 0 mM (○, △, □) and 100 mM (●, ▲, ■). The dotted line shows the PZC of HAP.

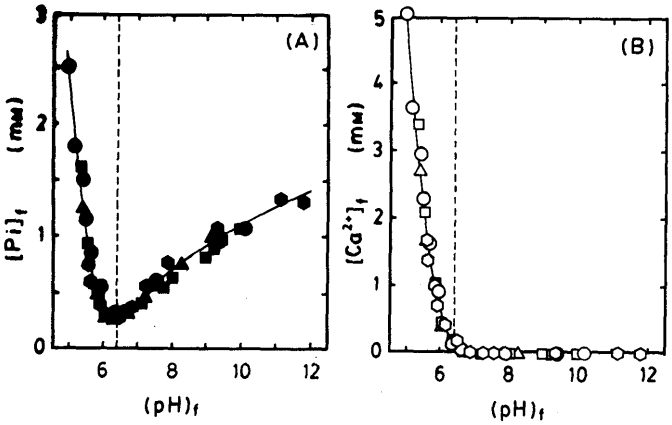

Fig. 4. Relationship between Equilibrium pH of the Solution and Equilibrium Concentrations of Pi (A) and Ca^{2+} (B) Released from HAP

Amino acids: none (●, ○), Asp (●, ○), Ala (▲, △) and Lys (■, □). Added concentration of KCl was 0 mM. The dotted line in each figure shows the PZC of HAP.

salt toward the HAP surface.[11] The adsorbed amount of Asp (X_{Asp}) decreased with an increase in $(pH)_f$ (Fig. 3A). Determination of X_{Asp} at pH lower than 4.6 was not done because HAP is a thermodynamically unstable phase below that pH.[12] When KCl (100 mM) was added to the solution, X_{Asp} (●) was smaller at low pH and slightly larger at high pH than that in the absence of KCl (○). In the case of the HAP–Ala system (Fig. 3B), the effects of pH and added KCl were rather small. On the other hand, X_{Lys} in the absence of KCl increased with $(pH)_f$ and then decreased, showing the maximum at pH 8.7 (Fig. 3C). When KCl was added to the solution, the dependence of X_{Lys} on $(pH)_f$ almost disappeared. The curves in Fig. 3A and those in Fig. 3C crossed at pH 6.4, which is the PZC of the HAP sample (see the dotted line).

In Figs. 4A and B, the concentrations of phosphate and calcium ions which were released from HAP are plotted against $(pH)_f$. It shows that $[Pi]_f$ decreased and then increased with $(pH)_f$. The break point corresponded to the PZC of HAP, where neither H^+ nor OH^- was adsorbed on HAP. On the other hand, $[Ca^{2+}]_f$ decreased monotonously with an increase in $(pH)_f$. Similar tendencies were also found for the KCl-containing systems, although the data are not shown here.

Effect of Added Salt on the Adsorption Behaviors

Phosphate ion was added as a mixture of KH_2PO_4 and K_2HPO_4 (0.74:0.26). This mixture was prepared to keep the solution pH at about the PZC of HAP. The results are shown in Figs. 5—7. When $CaCl_2$ was added to the HAP–AspNa system (Fig. 5A), both X_{Asp} and $X_{Ca^{2+}}$ (net increment of Ca^{2+} on the surface of HAP) increased with $[Ca^{2+}]_f$. The negative value in low concentrations of $[Ca^{2+}]_f$ means that the release of Ca^{2+} from HAP into the solution exceeded the adsorption of Ca^{2+} on HAP. When phosphate was added to the solution (Fig. 5B), X_{Asp} decreased with an increase in $[Pi]_f$. On the other hand, the

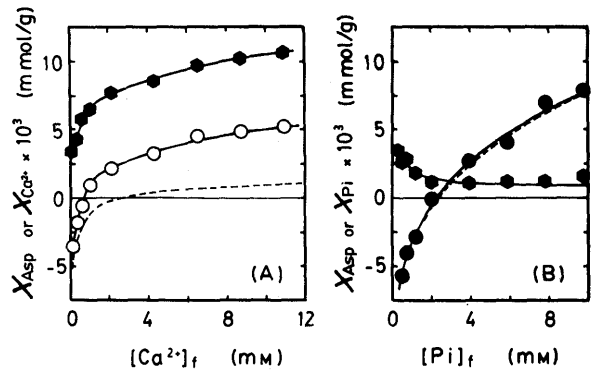

Fig. 5. (A) Effect of Addition of Ca^{2+} on the HAP–AspNa System. (B) Effect of Addition of Pi on the HAP–AspNa System

Initial concentration of AspNa was 1.6 mM. The dotted lines show the net increments of Ca^{2+} (A) and Pi (B) on the HAP surface in the absence of AspNa. ●: X_{Asp}, ○: $X_{Ca^{2+}}$, ●: X_{Pi}.

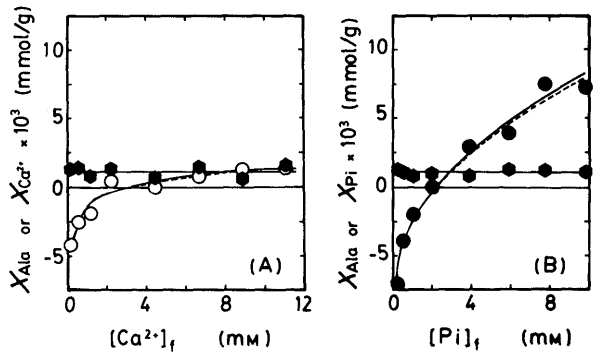

Fig. 6. (A) Effect of Addition of Ca^{2+} on the HAP–Ala System. (B) Effect of Addition of Pi on the HAP–Ala System

Initial concentration of Ala was 1.6 mM. The dotted lines show the net increments of Ca^{2+} (A) and Pi (B) on the HAP surface in the absence of Ala. ●: X_{Ala}, ○: $X_{Ca^{2+}}$, ●: X_{Pi}.

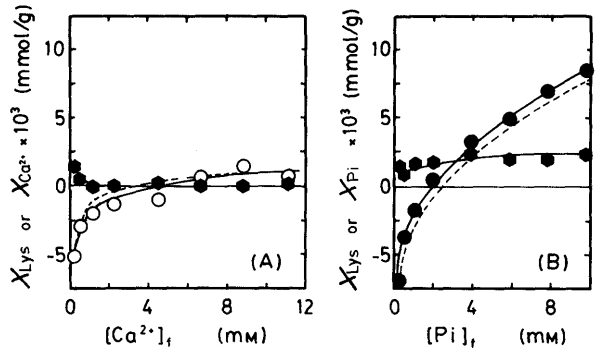

Fig. 7. (A) Effect of Addition of Ca^{2+} on the HAP–LysHCl System. (B) Effect of Addition of Pi on the HAP–LysHCl System

Initial concentration of LysHCl was 1.6 mM. The dotted lines show the net increments of Ca^{2+} (A) and Pi (B) on the HAP surface in the absence of LysHCl. ●: X_{Lys}, ○: $X_{Ca^{2+}}$, ●: X_{pi}.

adsorbed amount of Ala (X_{Ala}) was almost constant irrespective of the addition of the salts (Figs. 6A and B). In the case of the HAP–LysHCl system, X_{Lys} was decreased by the addition of Ca^{2+} (Fig. 7A) and slightly increased by the addition of Pi (Fig. 7B).

The dotted lines in Figs. 5—7 show the adsorbed amounts of Ca^{2+} (A) and Pi (B), in the absence of the amino acids. The deviations of the solid lines on the open or closed circles from the dotted lines correspond to the effect of the amino acids on $X_{Ca^{2+}}$ or X_{Pi}, respectively. It can be seen that $X_{Ca^{2+}}$ increased in the presence of Asp (Fig. 5A), and X_{Pi} did so in the presence of Lys (Fig. 7B).

Discussion

Amino acids are amphoteric and their net charges depend on the pH of aqueous media. Surface charges of HAP are also affected by the pH through protonation or deprotonation of Pi on the HAP surface and/or through adsorption or desorption of OH^-. These facts complicate the data analysis concerning the interaction between HAP and amino acids. The dissolution of HAP in acidic media has been extensively investigated as basic research on the mechanism of dental caries formation. HAP dissolves mainly in acidic solution to keep the solubility product of HAP (K_{sp}; $(Ca^{2+})^{10}(PO_4^{3-})^6(OH^-)^2$) constant. Therefore, the Ca/P ratio in solution becomes close to that in the bulk solid when the pH of the solution is decreased. As shown in Figs. 4A and B, $[Pi]_f$ and $[Ca^{2+}]_f$ increased with a decrease in $(pH)_f$ in the region below the PZC of HAP. On the other hand, in the region above the PZC, HAP showed a marked incongruent dissolution. This result can be explained in terms of the electroneutrality at the HAP surface and the restriction of K_{sp} of HAP in solution. That is, the negative charge resulting from the adsorption of OH^- repelled Pi from HAP and prevented the release of Ca^{2+}. The released Pi in the solution also limited the release of Ca^{2+}, because K_{sp} of HAP has to be kept constant.

When HAP was suspended in an aqueous solution of AspNa, Ala or LysHCl, the equilibrium pH of the solution ($(pH)_f$) was almost constant at the PZC of HAP and was independent of the adsorbed amount of the amino acid (Fig. 1B). This fact facilitates the discussion on the interaction between HAP and the amino acids based on the data shown in Figs. 1 and 2, because the effect of pH on the adsorbent and the adsorbate need not be considered. According to the dissociation constants of the amino acids ($pK_1 = 1.88$, $pK_2 = 3.65$ and $pK_3 = 9.60$ for Asp; $pK_1 = 2.34$ $pK_2 = 9.69$ for Ala; $pK_1 = 2.20$, $pK_2 = 8.90$ and $pK_3 = 10.28$ for Lys),[13] carboxylic and amino groups of the amino acids are virtually wholly ionized at pH around 6.45; hence, the net charges of Asp, Ala and Lys are -1, 0 and $+1$, respectively. In the previous papers,[14] it was reported that the adsorption of low-molecular-weight organic ions on HAP was accompanied by the release of the constituent ions of HAP with the same sign of the electric charge as the adsorbate. The increase of $[Pi]_f$ in the HAP–AspNa system and that of $[Ca^{2+}]_f$ in the HAP–LysHCl system were considered to occur primarily through the electrostatic repulsion with the adsorbed amino acids so as to maintain surface electroneutrality. On the contrary, Ala was adsorbed on HAP without the release of Ca^{2+} or Pi, because the net charge of Ala at that pH is approximately zero.

Figure 8 shows the activity product of $-\log (Ca^{2+})^{10}(PO_4^{3-})^6(OH^-)^2$, where parentheses mean the activity of the ion, as a function of the adsorbed amount of the amino acids. The values were calculated through a procedure similar to that described elsewhere.[14a,15,16] Here, (Ca^{2+}) was determined experimentally (Fig. 2C), because amino acids have weak chelating tendencies with divalent cations.[17-19] The calculated values of the activity product were within the range of literature values of $-\log K_{sp}$ of

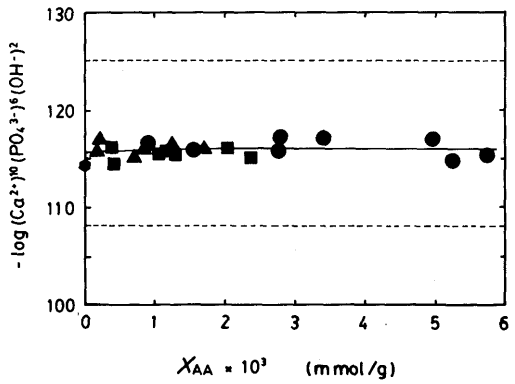

Fig. 8. Relationship between Adsorbed Amount of Amino Acids and Logarithm of Activity Product of HAP

Dotted lines show the upper and lower limits of the solubility product of HAP. Amino acids: ● none, ● Asp, ▲ Ala, and ■ Lys.

HAP and were almost constant irrespective of the adsorbed amount of the amino acids. The decreases of $[Ca^{2+}]_f$ (HAP–AspNa system) and $[Pi]_f$ (HAP–LysHCl system) can, therefore, be attributed to the increases of $[Pi]_f$ and $[Ca^{2+}]_f$, respectively, under the restriction of the K_{sp} of HAP.

When the solution pH deviates from the PZC of HAP (Figs. 3 and 4), the electric charges of the HAP surface and amino acids become important. In the region below the PZC, the surface of HAP carries positive charge (strictly speaking, the positive sites on HAP (Ca^{2+}) exceed the negative sites (Pi, OH^-) in number). However, even at pH 5.0, most of the Asp has a negative net charge of -1. Therefore, X_{Asp} increased with a decrease in $(pH)_f$ due to electrostatic attraction. On the other hand, when $(pH)_f$ becomes higher than the PZC, negative charge of the HAP surface repels that of Asp, resulting in the decrease in X_{Asp}. Similarly, X_{Lys} decreased with a decrease in $(pH)_f$ below the PZC, and it increased with an increase in $(pH)_f$ above the PZC. However, when $(pH)_f$ further increases, the species of Lys with a net charge of 0 and subsequently of -1 appear in the solution, and the concentration of cationic species of Lys (net charge $+1$) decreases. Therefore, X_{Lys} decreased after attaining the maximum around pH 8.7 with an increase in $(pH)_f$. When KCl was added to the solution, the electrostatic attraction and repulsion were screened. The intersections of the curves at the PZC of HAP in Figs. 3A and C reflect this fact. On the other hand, X_{Ala} had a low value at any given pH in the present work, because the effect of electrostatic attraction between Ala and HAP was insignificant. Moreno *et al.*[15] explained that the proximity of the oppositely charged groups at the α-position inactivated the zwitterion type of Ala (net charge 0), which is the dominant species below pH 9.7, as far as adsorption is concerned. The monovalent anion type of Ala, which appeared in alkaline media, is considered to have a chelating ability with Ca^{2+} on the HAP surface.[18a] However, negative charges of Ala and HAP prevented the adsorption of Ala in this region.

The facts mentioned above suggest that the participation of dissociable groups at the α-position of the amino acids for the adsorption process is not very significant. Dissociable terminal groups, the carboxylic group of Asp and the amino group of Lys, play important roles in the process. However, there are subtle differences between carboxylic and amino groups: Asp, which has a terminal carboxylic group, had a higher affinity than Lys at the PZC of HAP, and its affinity was less sensitive to the addition of KCl than that of Lys (Fig. 3). This result seems to be related to the following facts concerning the interaction between proteins and HAP. That is, the binding of acidic proteins to HAP is more specific than that of basic ones, which interact with HAP in a rather nonspecific electrostatic way[20]; some calcification-regulator proteins which have a high affinity to both HAP and Ca^{2+} have large contents of acidic amino acid residues.[3]

When Ca^{2+} or Pi was added to the solution, these ions were adsorbed on HAP. These adsorbed Ca^{2+} and Pi form charged sites on the HAP surface. Therefore, increases in X_{Asp} and $X_{Ca^{2+}}$ (Fig. 5A), and in X_{Lys} and X_{Pi} (Fig. 7B) were observed through synchronous adsorption. The value of X_{Asp} decreased with an increase in $[Pi]_f$ (Fig. 5A) and that of X_{Lys} did so with $[Ca^{2+}]_f$ (Fig. 7B). However, X_{Pi} and $X_{Ca^{2+}}$ seemed to be almost unaffected in the presence of Asp and Lys, respectively (compare the solid lines on the closed or open circles with the dotted lines). Such specific adsorption of the parent ions of HAP is essential for the crystal growth of HAP in body fluids containing various kinds of adsorbable components.[21]

In conclusion, amino acids are adsorbed on HAP electrostatically, and the adsorption affinity depends on the electrochemical conditions of the amino acids and of the HAP surface. Dissociative terminal groups of amino acids play important roles in the concurrent release of Ca^{2+} or Pi from HAP through adsorption. Asp has a high affinity at acidic pH, while Lys does so at basic pH. On the other hand, the affinity of Ala was low at any pH tested. Gorbunoff concluded from his chromatographic study that a slight variation in primary structure of proteins, especially with respect to acidic amino acids, affects the affinity to HAP, whereas a slight change in the tertiary structure is of little consequence.[20] The results of the present study are, therefore, considered to be important in relation to the affinity of proteins for HAP under various conditions.

References and Notes

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