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Inactivation of Human Alkaline Phosphatase by Sodium Thiocyanate[1)]

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The inactivation of human placental and intestinal alkaline phosphatases by sodium thiocyanate was investigated. Human placental alkaline phosphatase activity was completely inhibited by 2 M sodium thiocyanate, but human intestinal alkaline phosphatase was resistant to it. The structure of the inactivated enzyme was studied by means of the hydrophobic probe technique and circular dichroism method. Conformational changes had occurred in the secondary structure of the enzymes, and the hydrophobic regions of the inactivated enzymes had increased compared with those of the native enzymes. The addition of inorganic phosphate to the enzyme treatment system combined with sodium thiocyanate protected human placental alkaline phosphatase from inactivation, while human intestinal alkaline phosphatase was not protected. These findings suggest that the site attacked by sodium thiocyanate may be a region which induces conformational change upon binding of inorganic phosphate at the active site in human placental alkaline phosphatase.

Keywords——human tissue-specific alkaline phosphatase; sodium thiocyanate; hydrophobic region; secondary structure; conformational change

Alkaline phosphatase (AP, EC 3. 1. 3. 1) is a class of phosphomonoester hydrolase. Human tissue-specific AP can be classified into three isoenzymes: liver, intestinal and placental isoenzymes.[2-5)] It is very useful in diagnostic tests to distinguish each tissue-specific AP isoenzyme in sera by simple techniques, and various differential inhibitions in response to pH, certain amino acids, various reagents and metal ions have been utilized to determine these isoenzymes.[6)] However, practical techniques suitable for daily diagnostic analysis were not developed. Human placental AP (P-AP) in serum is a useful marker for malignancy and pregnancy,[7)] and human intestinal AP (I-AP) is significant in patients with hepatocirrhosis[8,9)] and chronic inflammatory bowel disease.[10)] Moreover, there are some similarities between P-AP and I-AP in amino acid compositions, consistent peptides, and so on.[2)] The two isoenzymes also share partially cross-reactive antigenic determinants.[2-5)] Thus, the differential determination of two isoenzymes is very useful. In the previous papers, we reported that the activities of AP isoenzymes were differently inhibited by raising of sodium thiocyanate (NaSCN) concentration, and the specific determination of P-AP and I-AP in serum could be performed by the combined use of anti-human tissue-specific AP antibodies and the above conditions.[11,12)] However, the reason for the difference of inhibitions of AP isoenzyme activities by NaSCN is not yet clear. In the present study, we investigated the difference of inhibitory effects of NaSCN on P-AP and I-AP proteins. The changes of protein structure of P-AP and I-AP induced by NaSCN were also investigated and the results are discussed.

Experimental

Materials——NaSCN was obtained from Nakarai Chemicals, Ltd. 8-Anilino-1-naphthalene sulfonate (ANS), obtained from Tokyo Kasei Co., Ltd., was recrystallized three times from hot water with Norit treatment. Other reagents used were of the highest purity commercially available.

Enzymes——The purifications of human liver AP (L-AP), P-AP and I-AP were performed as previously described.[13-15] The homogeneity of the purified enzymes was demonstrated by disc polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE).

Assay of AP Activity——AP activity was determined by the method of Kind and King.[16]

Assay of Protein——Protein was determined by the method of Lowry *et al.*[17] using bovine serum albumin as a standard, and the absorbance at 280 nm was also measured.

Inactivation of the Enzyme——One mg of the purified enzyme was dissolved in 10 mM Tris-HCl buffer (pH 7.4). An equal volume of NaSCN in the above buffer was added to the enzyme solution. After the enzyme solution had been incubated with NaSCN at 37 °C for 30 min, the protein was separated from NaSCN by gel filtration on a Sephadex G-25 column (1.5 × 40 cm) equilibrated with the same buffer as used for the inactivation of the enzyme. The enzymes thus treated were used for the polyacrylamide gel electrophoresis experiments, fluorescence measurements and circular dichroism measurements.

PAGE and SDS-PAGE——PAGE was carried out with 7.5% polyacrylamide gel according to the method of Davis,[18] and SDS-PAGE was carried out with 7.5% polyacrylamide gel according to the method of Weber and Osborn.[19] Proteins were stained with Coomassie Brilliant Blue R-250.

Fluorescence Measurement——Fluorescence measurements were made with a Shimadzu RF-510 fluorescence spectrophotometer equipped with a recorder. The temperature was controlled at 25 °C by means of a thermostatically controlled cell holder. The excitation wavelength was 380 nm, and the fluorescence emission was recorded between 400 and 600 nm. Fluorescence measurements were taken at least 30 min after the incubation.

Circular Dichroism——Circular dichroic (CD) spectra were measured with JASCO J-40S automatic recording spectropolarimeter. Recordings of CD spectra were taken in the 200 to 320 nm region. The enzymes were dialyzed against 2 mM Tris-HCl buffer (pH 7.4). The protein concentrations used for CD spectra were 1.9×10^{-7} M (I-AP) and 2.5×10^{-7} M (P-AP). The temperature was controlled at 20 °C by means of a thermostatically controlled cell holder.

Results

Inhibitory Effect of NaSCN on AP Activity

The effect of NaSCN on AP activity was investigated. As shown in Fig. 1, L-AP and P-AP activities were completely inhibited by treatment with 2 M NaSCN at 37 °C for 30 min, while I-AP was resistant to 1—3 M NaSCN treatment. This inhibition was irreversible and differed from the inorganic phosphate inhibition (competitive inhibition). Therefore, NaSCN inhibition was apparently due to the denaturation of AP protein. Then, the differential inactivation of P-AP and I-AP with NaSCN was investigated. The above finding was supported by the results that the activation energies of native APs (P-AP 12.3 kcal/mol, I-AP 14.2 kcal/mol) were consistent with those of the NaSCN-treated enzymes (P-AP 12.6 kcal/mol, I-AP 14.0 kcal/mol). However, only P-AP was protected by the addition of inorganic phosphate from NaSCN inactivation as shown in Fig. 2. P-AP and I-AP were not protected from inactivation by the addition of L-phenylalanine to the treatment system with NaSCN.

P-AP and I-AP Protein Inactivations by NaSCN

Figure 3 shows PAGE patterns of the NaSCN inactivated enzymes. The native AP protein band and additional protein bands were observed, and the number of additional protein bands in the case of P-AP increased with increase of the NaSCN concentration. When inorganic phosphate was added to the treatment system with NaSCN, however, the additional protein bands were not found (gel A-3). This result indicates that P-AP was protected by inorganic phosphate, and this is in agreement with the result of the test on the protection of P-AP activity shown in Fig. 2.

In the case of I-AP, as shown in Fig. 3, no additional protein bands of I-AP were observed on treatment with 1 M NaSCN, but additional protein bands were found on

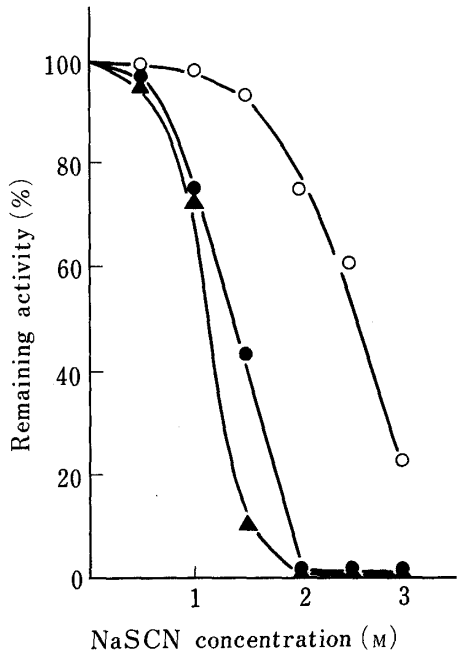

Fig. 1. Effect of NaSCN on AP Activity

One hundred μl of the purified enzyme (1 mg/ml) was incubated with an equal volume of NaSCN in 10 mM Tris-HCl buffer (pH 7.4) at 37 °C for 30 min. After the incubation, the mixture was diluted 100-fold with the above buffer, and the remaining activity was assayed. —●—, P-AP; —○—, I-AP; —▲—, L-AP.

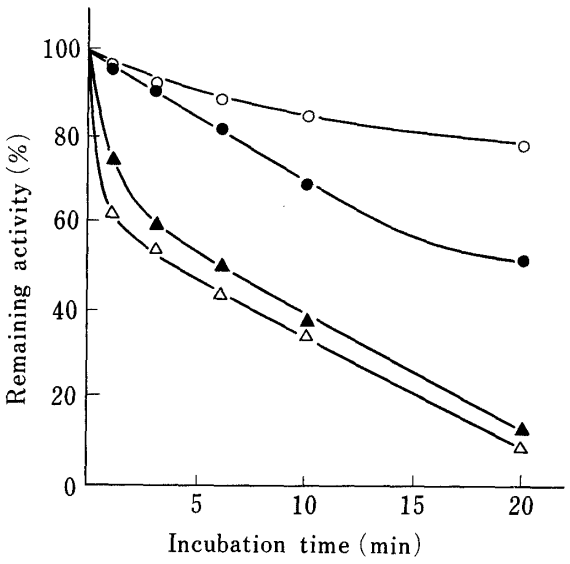

Fig. 2. Effect of Inorganic Phosphate on Inhibition of AP Activity by NaSCN

One hundred μl of the purified P-AP and I-AP (1 mg/ml) was incubated at 37 °C with an equal volume of NaSCN in 10 mM Tris-HCl buffer (pH 7.4). After the incubation, the mixture was diluted 100-fold with the above buffer, and the remaining activity was assayed. —●—, P-AP with 1.5 M NaSCN; —○—, P-AP with 1.5 M NaSCN and 10 mM Na_2HPO_4; —▲—, I-AP with 3.5 M NaSCN; —△—, I-AP with 3.5 M NaSCN and 60 mM Na_2HPO_4.

treatment with 2 or 3 M NaSCN. Gel B-3 shows that I-AP was not protected by inorganic phosphate from inactivation. This result was in agreement with the result regarding the inhibitory effect of NaSCN on I-AP activity, as shown in Fig. 2. The AP activity on the gel coincided with the native protein band of P-AP and I-AP. Therefore, it is considered that the additional protein bands were inactivated enzyme protein bands.

On the other hand, P-AP and I-AP treated with 1, 2 and 3 M NaSCN each gave a single protein band in SDS–PAGE (not shown). These results suggest that the inactivated enzymes were aggregated forms of the native enzymes. In the case of P-AP treated with 2 or 3 M NaSCN, low-molecular-weight protein bands were found in gels A-4 and -5. It is considered that the protein bands were the monomer form of the native enzyme.

Fluorescence Spectra of ANS–AP Complexes

ANS has been employed to investigate the protein structure of enzymes, particularly hydrophobic regions of proteins. Fluorescence spectra of ANS–AP complexes excited at 380 nm are shown in Fig. 4. The fluorescence spectrum of ANS itself in aqueous solution exhibited an emission maximum at 515 nm. The emission maximum of ANS, when excited in the presence of the enzymes, shifted to 473 nm. This may indicate that ANS was bound to the hydrophobic regions of the enzymes.[20,21] On treatment with NaSCN, the hydrophobic regions of the inactivated enzymes increased compared with those of the native enzymes. When P-AP was treated with 1 M NaSCN and 0.1 M inorganic phosphate, the fluorescence intensity at 473 nm of ANS did not differ from that of ANS bound to the native enzyme. This result is consistent with the protection of P-AP by inorganic phosphate from inactivation, as shown in Figs. 2 and 3, and suggests that a region of P-AP which influences the activity was attacked by 1 M NaSCN. The fluorescence intensity of ANS bound to I-AP treated with 1 M NaSCN increased compared with that of ANS bound to the native enzyme, though I-AP

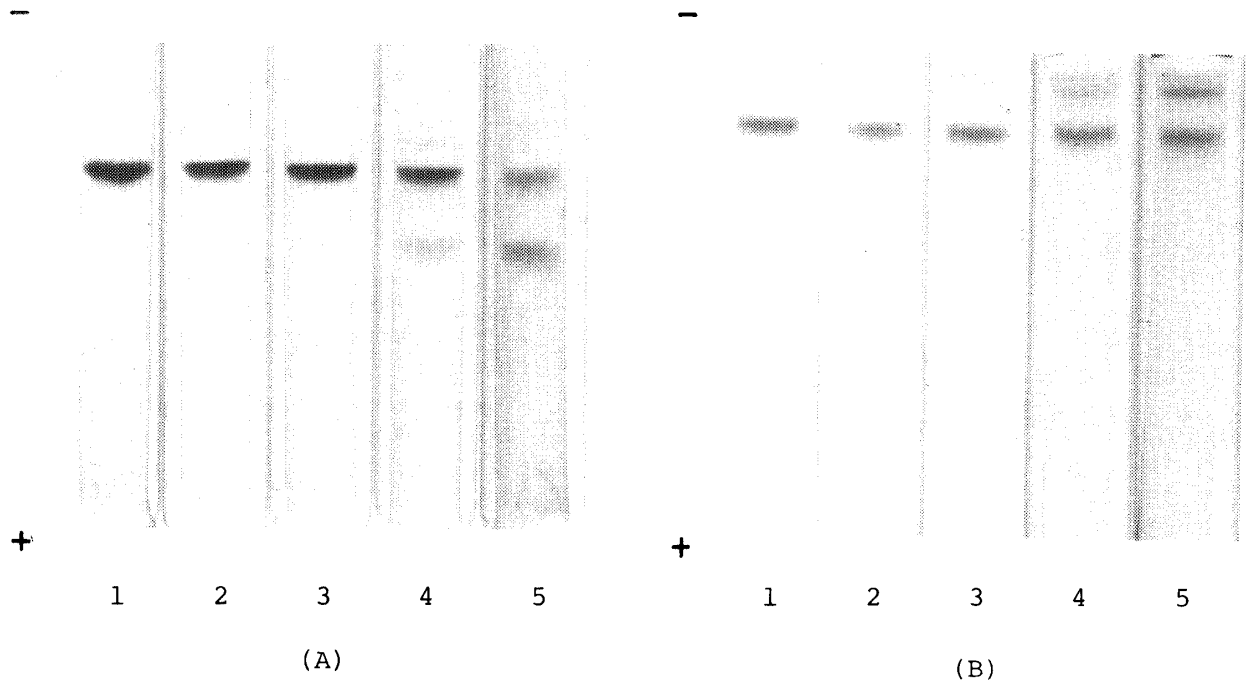

Fig. 3. Disc Polyacrylamide Gel Electrophoretic Patterns of Inactivated P-AP and I-AP by NaSCN

(A) P-AP, (B) I-AP. gel 1, native enzyme; gels 2, 4 and 5, enzyme inactivated by 1, 2 and 3 M NaSCN, respectively; gel A-3, P-AP inactivated by 1 M NaSCN and 0.1 M Na_2HPO_4; gel B-3, I-AP inactivated by 2 M NaSCN and 0.1 M Na_2HPO_4. The remaining activity of P-AP treated with 1 M NaSCN and 0.1 M Na_2HPO_4 was 94.1% of the original activity, while that of P-AP treated with 1 M NaSCN was 77.8% of the original activity. The remaining activity of I-AP treated with 2 M NaSCN and 0.1 M Na_2HPO_4 was 78.5% of the original activity, while that of I-AP treated with 2 M NaSCN was 80.2% of the original activity. Other conditions were as detailed in Materials and Methods.

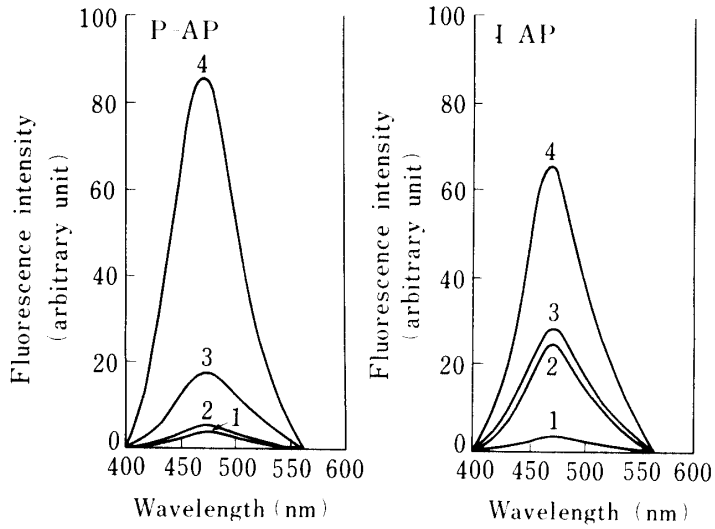

Fig. 4. Fluorescence Spectra of ANS–AP Complexes

The incubation medium consisted of 3.9×10^{-5} M ANS and 2.6×10^{-7} M enzyme protein in 10 mM Tris-HCl buffer (pH 7.4). 1, native enzyme; 2, enzyme inactivated by 1 M NaSCN and 0.1 M Na_2HPO_4; 3, enzyme inactivated by 1 M NaSCN; 4, enzyme inactivated by 3 M NaSCN. Other conditions were as detailed in Materials and Methods.

activity was not inhibited. When I-AP was treated with 1 M NaSCN and 0.1 M inorganic phosphate, the fluorescence intensity of ANS bound to the enzyme was the same as that of ANS bound to the enzyme treated with 1 M NaSCN, though the full activity remained. The

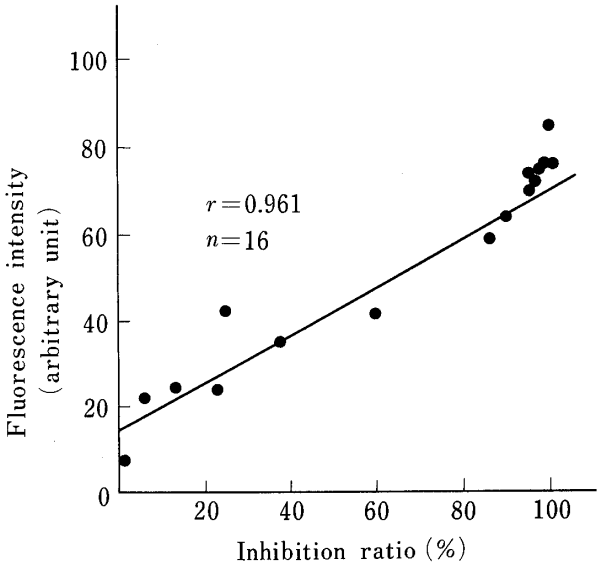

Fig. 5. Correlation between Inhibition of Activity and Fluorescence Intensity of ANS–P-AP Complexes

The conditions were as detailed in the legend to Fig. 4 and in the text.

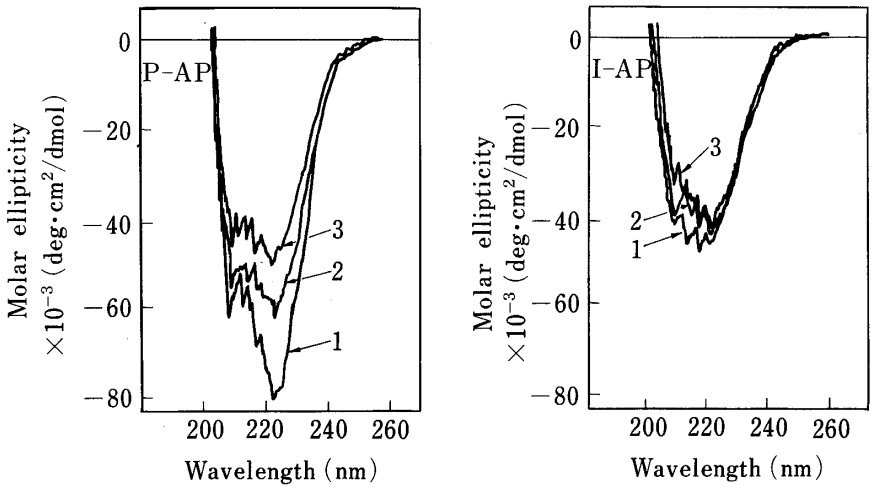

Fig. 6. Far Ultraviolet Circular Dichroic Spectra of P-AP and I-AP

1, native enzyme; 2, enzyme inactivated by 1 M NaSCN and 0.1 M Na_2HPO_4; 3, enzyme inactivated by 1 M NaSCN. Other conditions were as detailed in Materials and Methods.

above findings suggest that the denaturation of a region in I-AP attacked by 1 M NaSCN has no influence on the activity.

The correlation between the inhibition of P-AP activity by NaSCN and the increase of fluorescence intensity of ANS–P-AP complexes was investigated. As shown in Fig. 5, the correlation coefficient was excellent ($r=0.961$). This result suggests that the hydrophobic region of P-AP increases with increasing concentration of NaSCN, and the activity is inhibited.

CD Spectra of Inactivated Enzymes

As shown in Fig. 6, the ultraviolet (UV) CD spectra of P-AP and I-AP were used as reference spectra for the native state of the backbone or secondary structure of the enzymes. In the spectra of both P-AP and I-AP, negative bands occurred at 208 and 222 nm. Changes in these bands on treatment of the enzymes with NaSCN represent evidence of induced change in the secondary structure of the enzymes.[22] In particular, the treatment of P-AP with 1 M NaSCN caused large alterlation in the far UV CD spectrum relative to that of the native P-

AP. The decrease in the negative CD band at 222 nm shown in Fig. 6 suggests that conformational change has occurred in the secondary structure of the enzyme. In the presence of inorganic phosphate during treatment of P-AP with 1 M NaSCN, the far UV CD spectrum of this enzyme indicates that a slight conformational change had occurred in the secondary structure of the enzyme. This result is consistent with the finding that P-AP activity was protected by inorganic phosphate from inactivation. On treatment of I-AP with 1 M NaSCN or 1 M NaSCN and 0.1 M inorganic phosphate, however, the far UV CD spectrum of the enzyme was the same within experimental error as the native one, indicating that little conformational change had occurred in the secondary structure of the enzyme. This result is consistent with the finding that I-AP activity was not inhibited by treatment with 1 M NaSCN.

Discussion

P-AP was irreversibly inactivated by NaSCN, while I-AP was resistant to it. The hydrophobicity of the inactivated enzymes was increased compared with those of the native enzymes, and inactivated protein bands were found in the PAGE patterns. However, SDS–PAGE patterns of both inactivated enzymes gave a single protein band, suggesting that the inactivated enzyme consisted of the monomer form and the forms aggregated by hydrophobic interaction.[23,24] It is considered that the inactivation of the enzyme treated with NaSCN was caused by conformational change occurring in the secondary structure of the enzyme. Considering that P-AP was protected by inorganic phosphate from NaSCN inactivation, the site attacked by NaSCN might be a specific region involved in the activity. Daemen and Riordan showed that inorganic phosphate protected the enzyme against inhibition by inhibitors.[26] It is well known that the active sites of liver, intestinal and placental APs are very similar,[2] and also that the amino acid sequences around the active site in APs from *E. coli* and several mammalians tissues are similar to each other.[27–31] Therefore, it is suggested that the site attacked by NaSCN is not the active site of P-AP, but is probably a specific hydrophilic region which influences the activity by the binding of phosphate to the active site as described by Sussman.[25] On the other hand, though P-AP activity was not inhibited by treatment with 1 M NaSCN and 0.1 M inorganic phosphate, the negative CD band at 222 nm of this enzyme was slightly decreased compared with that of the native one. This result indicates that a region in P-AP which has no influence on the activity was also attacked by 1 M NaSCN.

In the case of I-AP, the enzyme was resistant to 1 M NaSCN, but the fluorescence intensity of ANS bound to the enzyme was increased compared with that of ANS bound to the native enzyme. Also, the presence of inorganic phosphate did not protect the enzyme from inactivation with NaSCN. The above findings suggest that a region in I-AP which has no influence on the activity was probably attacked by low concentrations of NaSCN. Since the binding of inorganic phosphate to the active site induces a conformational change in the enzymes, the structure of the region may be very rigid, so that I-AP escapes inactivation by low concentrations of NaSCN. However, at high concentrations of NaSCN, various regions in the enzyme may be nonspecifically denatured, leading to loss of the activity.

References and Notes

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