Dedicated to Prof. N. Shimazono on his 70th birthday.

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The Interaction between Xanthurenic Acid-Insulin Complex and Zinc Ions[1,2]

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An equimolar mixture of a xanthurenic acid-insulin complex and $ZnSO_4$ was separated into an insulin peak and a peak containing xanthurenic acid (XA) and Zn by Sephadex G-75 column chromatography. XA and di-[L-histidino]-zinc (II) readily combined to produce di-[L-histidino]-di-xanthurenato zinc (II) (His_2-Zn^{2+}-XA_2). By increase in the concentration of Zn^{2+} ions, XA was removed from di-[L-histidino]-di-xanthurenato zinc (II) (His_2-Zn^{2+}-XA_2) as XA-Zn^{2+}. The XA-insulin complex showed decreased relative intensity of fluorescence compared with the Zn-insulin when excited at a wavelength of 284 nm. The difference spectrum between native Zn-insulin and XA-insulin complexes showed a slight red shift. A difference in the CD spectrum between native Zn-insulin and XA-insulin complexes was observed.

Since 1950, Kotake and Inada (*1, 2*) have been studying the physiological activity of XA and have found that XA, a metabolite of tryptophan, has a diabetogenic action. In 1968, Murakami (*3*) found that XA and insulin combine readily when mixed in buffer solutions or sera. The physiological activity of the XA-insulin complex was shown to be weaker than that of native Zn-insulin in various ways, such as by comparison of the effects of the two forms of insulin on the blood glucose level and on glucose uptake by the diaphragm (*4*). Kotake *et al.* (*5*) reported that the decreased hormonal activity of the XA-insulin complex regained its activity on addition of Zn^{2+} ions. The present work was conducted to clarify why insulin shows decreased activity in the XA-insulin complex and to analyze the interaction between the XA-insulin complex and zinc ions. This investigation was expected to provide valuable information about the active site of insulin and about the biochemical aspects of the etiology of experimental diabetes mellitus.

MATERIALS AND METHODS

Chemicals—All chemicals were of the best analytical grade obtainable from Yoneyama Chemical Industries, Ltd. and Nakarai Chemicals, Ltd.

[1] Dedicated to Professor Dr. Norio Shimazono on his 70th birthday.

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Sephadex G-75 was purchased from Pharmacia Fine Chemicals. Crystalline ox Zn-insulin was purchased from the Connaut Laboratory (Canada). The XA-insulin complex was prepared by the method of Murakami (*3*). Di-[L-histidino]-zinc (II) was synthesized according to the method of Kretsinger *et al.* (*6*).

Chromatographic Methods—Sephadex G-75 chromatography was performed on a 2×57 cm column previously equilibrated with 0.5 M Tris-HCl buffer (pH 8.1). Elution was carried out with the same buffer at a flow rate of 35 ml/h and 3 ml fractions were collected.

The Determination of the Composition of the Complexes—The compositions of the di-[L-histidino]-zinc (II)-XA complex and the XA-Zn^{2+} complex were determined by the continuous variation method (*7*).

Spectrophotometric Measurements—The spectra of the native Zn-insulin and XA-insulin complexes were obtained by using 1 cm path-length quartz cuvettes. The absorption spectra of XA were measured with a Union Giken high sensitivity spectrophotometer, SM 401. The fluorescence spectra of insulin were studied with a Hitachi fluorospectrophotometer and Union Giken fluorospectrophotometer, FS 301.

The Measurement of Zn Concentration—Zn concentration was measured with a Hitachi atomic absorption spectrometer, 208, at 2137.5–8 Å.

The Measurement of the Circular Dichroism Spectra—CD measurements were made using a Union Giken CD spectropolarimeter. Instrument performance was tested by measuring *d*-10-camphorsulfonic acid.

RESULTS

Interaction of Metal Ions with XA—Figure 1 shows the gel filtration chromatography of XA and $ZnSO_4$. The equimolar mixtures of XA and $ZnSO_4$ were eluted with 0.5 M Tris-HCl buffer (pH 8.1).

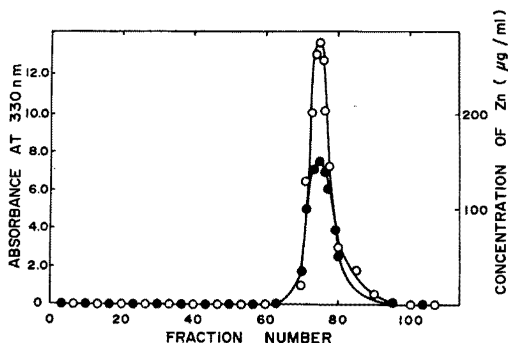


Fig. 1. Sephadex G-75 column chromatography of XA and $ZnSO_4$. 50 μmol of XA and $ZnSO_4$ were dissolved in 0.5 M Tris-HCl buffer (pH 8.1) under the conditions described in the text. ○, XA absorbance at 330 nm; ●, Zn concentration.

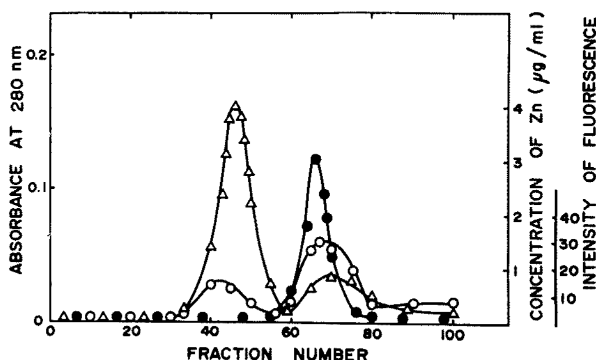


Fig. 2. Sephadex G-75 column chromatography of XA-insulin complex and $ZnSO_4$. 0.815 μmol of XA-insulin complex and $ZnSO_4$ were dissolved in a 0.5 M Tris-HCl buffer (pH 8.1). △, Absorbance rate=280 nm; ●, Zn concentration; ○, intensity of fluorescence. The XA fluorescence was measured at 430 nm against excitation at 365 nm.

XA was eluted in the same fractions as the Zn^{2+} ions. This result indicated the possibility of complex-formation. An equimolar mixture of the XA-insulin complex and $ZnSO_4$ in a 0.5 M Tris-HCl buffer was also applied to a Sephadex G-75 column (Fig. 2).

The first peak of 280 nm absorbance was insulin. The second peak was very close to that of XA seen in Fig. 1. The intensity of fluorescence also was measured at 430 nm by excitation at 365 nm to determine the peak of XA. Zn^{2+} ions were eluted in the same fractions as XA. These results suggest that Zn^{2+} ions may remove XA from the XA-insulin complex. In order to examine the mechanism more closely, di-[L-histidino]-zinc (II) was used to analyze the binding site of Zn^{2+} and XA on the insulin molecule, because Murakami (*3*) had reported that XA probably bound to the imidazole group of the histidine residue in the insulin molecule, and Hodgkin (*8*) reported that Zn bound to the imidazole group of the histidine residue in insulin from X-ray diffraction analysis. This complex was therefore used to analyze the interaction between XA and the histidine residue in the insulin molecule to locate the binding site of zinc in the molecule and to observe the mechanism of XA release from the XA-insulin complex by the addition of Zn^{2+} ions. The di-[L-histidino]-zinc (II) and XA were dissolved in 0.5 M Tris-HCl buffer (pH 8.1). The total concentration of the di-[L-histidino]-zinc (II) and XA was 1.0×10^{-4} M. The ultraviolet absorption of XA was measured at several mole-fractions of XA to the di-[L-histidino]-zinc (II) complex, and ΔA, the deviation of the absorbance of the mixed solution from the sum of the absorbances of the components, was plotted against the mole-fraction as shown in Figs. 3 and 4.

The plots in Fig. 3 show a peak at the mole-fraction of 0.66, indicating the molar ratio of the di-[L-histidino]-zinc (II) to XA was 1 : 2, and that of histidine, Zn^{2+} and XA was 2:1:2.

Figure 4 shows the continuous variation plots of the XA and Zn^{2+} complexes at several mole-fractions of XA. At 365.5 nm, the curve shows a peak at the mole-fraction of 0.5, indicating the molar ratio of XA to the Zn^{2+} ions was 1:1. The absorption spectrum of XA did not show deviation at 343 nm in 0.1 M acetate buffer (pH 5.4), implying that XA does not form a complex with zinc ions in acidic solution.

Effect of Zn^{2+} Ions on the Absorption Spectra of the Complexes—The XA-Zn^{2+} and His_2-Zn^{2+}-XA_2 complexes gave markedly different absorption spectra. The His_2-Zn^{2+}-XA_2 complex had a broad peak at 368.50 nm, while the XA-Zn^{2+} complex had a peak at 370 nm. When equimolar solutions of the complexes were each added to a Zn^{2+} ion solution of ten times higher concentration, the resulting

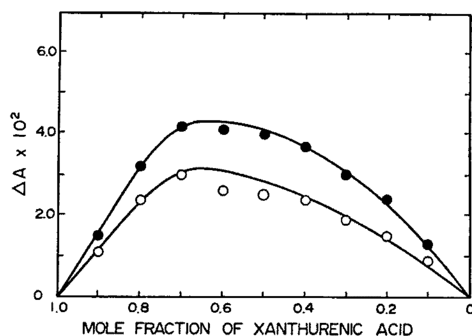


Fig. 3. The formation of the complex of di-[L-histidino]-zinc (II) and XA. The curves show the continuous variation plots for the reaction of di-[L-histidino]-zinc (II) and XA at 25°C. The total concentration of di-[L-histidino]-zinc (II) and XA was 1.0×10^{-4} M. The spectrophotometric measurements were conducted in 0.5 M Tris-HCl buffer (pH 8.1). ○, ΔA at 328.5 nm; ●, ΔA at 366 nm.

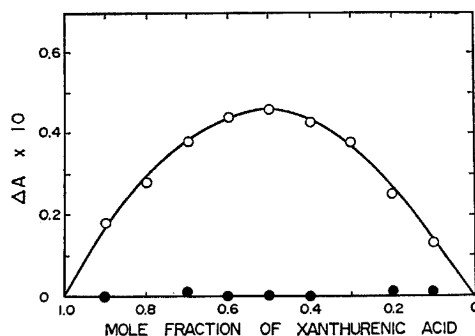


Fig. 4. The formation of the complex of XA and Zn^{2+} ion. The curves show the continuous variation plots for the reaction of XA and Zn^{2+} ion at 25°C. The total concentration of XA and Zn^{2+} ions was 1.0×10^{-4} M. The spectrophotometric measurements were conducted in 0.5 M Tris-HCl buffer (pH 8.1) and 0.1 M acetate buffer (pH 5.4). ○, ΔA at 365.5 nm measured in 0.5 M Tris-HCl buffer (pH 8.1); ●, ΔA at 343 nm measured in 0.1 M acetate buffer (pH 5.4).

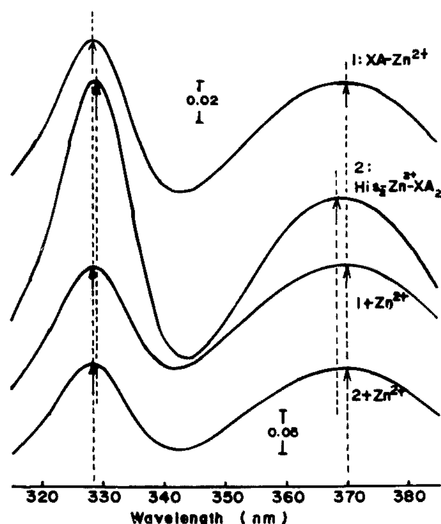


Fig. 5. Complex 1 = Xanthurenic acid-Zn^{2+}; complex 2 = di-[L-histidino]-di-xanthurenato zinc (II). The upper two spectra are for 5×10^{-5} M complex 1 and complex 2 solutions in 0.5 M Tris-HCl buffer (pH 8.1). The lower two spectra are for 3.3×10^{-5} M solutions of complexes 1 and 2 in 0.5 M Tris-HCl buffer (pH 8.1) with 3.3×10^{-4} M Zn^{2+} ions. The spectra were measured with a Union Giken Spectrophotometer, SM 401, at a slit width of 0.50 mm and a temperature of 25°C.

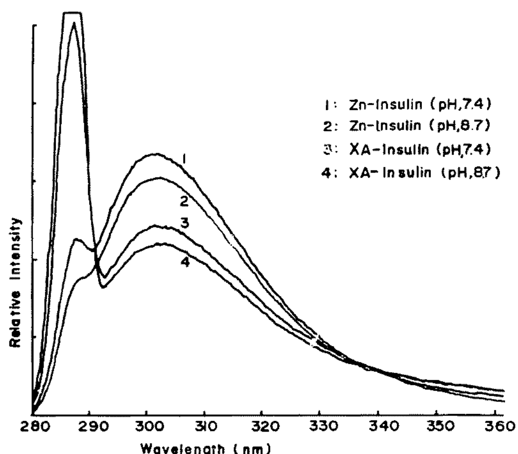


Fig. 6. Fluorescence spectra of Zn-insulin and XA-insulin complexes. Fluorescence spectra of 4.17×10^{-5} M solutions of native Zn-insulin and XA-insulin complexes in 0.5 M Tris-HCl buffers (pH 7.4 and 8.7, respectively). Fluorescence spectra were measured with a Union Giken fluorospectrophotometer, FS 301, at an excitation of 284 nm and a slit width of 7 nm. Emission was followed from 280 to 380 nm at slit width of 3.5 nm.

solutions displayed the same absorption spectrum. Comparison of the four spectra (Fig. 5) indicates that the His_2-Zn^{2+}-XA_2 complex was converted to the XA-Zn^{2+} complex with the increase of the concentration of Zn^{2+} ions, by removal of XA from di-[L-histidino]-di-xanthurenato zinc (II) (His_2-Zn^{2+}-XA_2).

Spectrophotometric Properties of the XA-Insulin Complex—Figure 6 shows the fluorescence spectra of the Zn-insulin and the XA-insulin complexes, which have a large peak at 300 nm. The fluorescences of Zn-insulin and XA-insulin were measured in 0.5 M Tris-HCl buffers of pH 7.4 and pH 8.7, respectively. The excitation was made at the wavelength of 284 nm in order to observe tyrosine residues of insulin, according to Labeyrie *et al.* (*9*).

The peaks at 285 nm preceding the 300 nm peaks were due to dispersed light. The intensity of fluorescence decreased in the case of the XA-insulin complex. The absorption spectra of the native Zn-insulin and XA-insulin complexes differed greatly. The difference spectrum showed a slight red shift (Fig. 7).

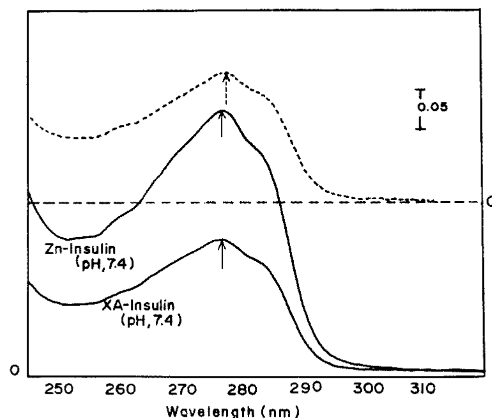


Fig. 7. The absorption spectra of native Zn-insulin and XA-insulin complexes. Ultraviolet spectra of 1.39×10^{-5} M solutions of the native Zn-insulin and XA-insulin complexes in 0.5 M Tris-HCl buffer (pH 7.4) were obtained with a Union Giken Spectrophotometer, SM 401.

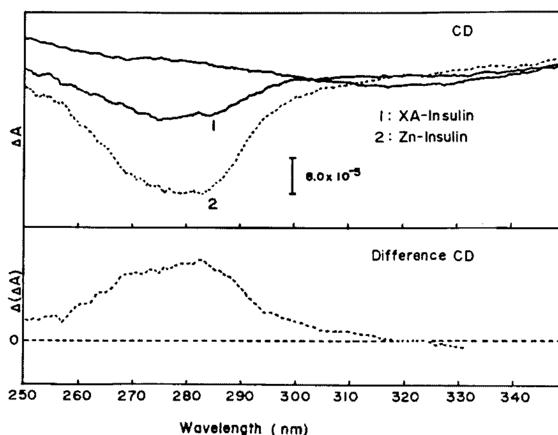
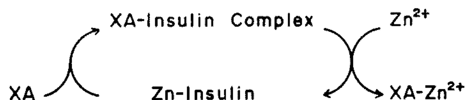


Fig. 8. Circular dichroism spectra of native Zn-insulin and XA-insulin complexes. The measurements were made with a Union Giken CD spectropolarimeter at 25°C on 4.17×10^{-5} M solutions of native Zn-insulin and XA-insulin complexes in 0.5 M Tris-HCl buffer (pH 7.4) and standard *d*-10-camphorsulfonic acid, respectively.

The circular dichroism of the XA-insulin and Zn-insulin complexes was studied in 0.5 M Tris-HCl buffer (pH 7.4). A difference in CD spectrum was seen between Zn-insulin and the XA-insulin complex, which might indicate a conformational change induced by XA (Fig. 8).

DISCUSSION

The observation that zinc ions moved with XA on Sephadex G-75 column chromatography indicated that the zinc ions combined with the XA. Also, when equimolar mixture of the XA-insulin complex and $ZnSO_4$ were applied to the Sephadex G-75 column and eluted with 0.5 M Tris-HCl buffer (pH 8.1), the chromatographic profile showed that the XA-Zn^{2+} complex was removed from the XA-insulin complex. From these results and from those of the experiment using the di-[L-histidino]-di-xanthurenato zinc (II) (His_2-Zn^{2+}-XA_2), it is clear that this complex was transformed to XA-Zn^{2+} when the concentration of zinc ion was increased. This His_2-Zn^{2+}-XA_2 complex is useful for analyzing the mechanism by which the activity of the XA-insulin complex is restored. In the presence of XA, Zn-insulin readily forms a complex with XA. Formation of the XA-insulin complex reduces the physiological activity of insulin. This complex is transformed into Zn-insulin and XA-Zn^{2+} in the presence of zinc ions. Zn-insulin and XA-insulin complexes form a cycle in this circumstance.



The fluorescence spectra of the Zn-insulin and XA-insulin complexes showed decreased intensity of the XA-insulin complex. The decrease in relative intensity of the fluorescence and the difference absorption spectra (Figs. 6 and 7) of the XA-insulin complex indicate that there were some changes in the vicinity of the tyrosine residue and also suggest that XA was located in the vicinity of this residue and had some effect on it. The results of the study of the CD spectra of the Zn-insulin and XA-insulin complexes suggested that the XA bound to the Zn-insulin molecule might produce some conformational change.

The present study revealed that the XA-insulin complex regained its activity on addition of zinc ions; the conformational changes induced by XA might reduce the physiological activity of insulin.

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