Studies on D-Glucose-isomerizing Enzyme from *Bacillus coagulans*, Strain HN-68

Part VI. The Role of Metal Ions on the Isomerization of D-Glucose and D-Xylose by the Enzyme

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A functional role of Co^{2+} and Mn^{2+} in the D-glucose- and D-xylose-isomerizing reactions by D-glucose-isomerizing enzyme obtained from the cells of *Bacillus coagulans*, strain HN-68 was investigated. (1) The enzyme required Co^{2+} and Mn^{2+} for D-glucose- and D-xylose-isomerizing activities, respectively. (2) The enzyme which bound the metal, Co^{2+} - or Mn^{2+} -enzyme, was active form. Co^{2+} was bound to the enzyme in a molar ratio of 4:1. (3) The rate of activation by metal ion varied with incubation pH. (4) The binding of substrate to the enzyme was completely independent in the presence of metal ions. (5) However, it seemed unlikely that the Co^{2+} and Mn^{2+} acted as catalyzer on the reaction. (6) The binding sites for Co^{2+} and Mn^{2+} were different from each other. (7) The experimental data obtained might be successfully explained in terms of the suitable conformational changes for D-glucose and D-xylose isomerization, which were induced in the catalytic sites of the enzyme by binding Co^{2+} and Mn^{2+} , respectively.

In the previous paper,^{1,2)} the author has reported some of the enzymatic and physico-chemical properties of D-glucose-isomerizing enzyme which was prepared as crystalline form from the cells of *Bacillus coagulans*, strain HN-68. It has been proved that three activities of D-glucose, D-xylose and D-ribose isomerization occurred at the same active site of the enzyme molecule and that the enzyme may be considered to be D-xylose isomerase (D-xylose ketol-isomerase, EC 5.3.1.5). The enzyme showed following interesting properties with regard to relationship between kind of metal ion added and substrate specificity: D-glucose- and D-ribose-isomerizing activities of the enzyme are specifically stimulated by

Co^{2+} , while D-xylose-isomerizing activity by Mn^{2+} . The present paper describes the functional role of Co^{2+} and Mn^{2+} on the D-glucose- and D-xylose-isomerizing activities of the enzyme.

MATERIALS AND METHODS

Metal-free D-glucose-isomerizing enzyme. D-Glucose-isomerizing enzyme crystallized twice was prepared from the cells of *Bacillus coagulans*, strain HN-68, by the method described in previous paper.¹⁾ In order to remove metallic dication, the crystalline enzyme was dissolved in 0.01 M EDTA and dialyzed successively against 500 volume of 0.01 M EDTA and of distilled water. This preparation obtained showed no activities of the isomerization without adding mental ion.

Protein concentration. Amount of protein was

1) G. Danno, *Agr. Biol. Chem.*, **34**, 1795 (1970).

2) G. Danno, *ibid.*, **34**, 1805 (1970).

measured spectrophotometrically at $280\text{ m}\mu$ with an extinction coefficient of 10.6 for 10 mg of enzyme per ml, in 0.1 M acetate buffer (pH 6.0) and a 10 mm light path.¹⁾

Assay of enzyme activities. For the assay of D-glucose-isomerizing activity, the mixture containing 1 ml of enzyme solution and 1 ml of 0.03 M CoCl_2 solution was preincubated for 20 min at 40°C . To the mixture was added one ml of 0.3 M D-glucose solution in 0.02 M barbital buffer (pH 7.0) and continued the incubation at 40°C for 30 min. For the assay of D-xylose-isomerizing activity, the mixture containing 1 ml of enzyme solution and 1 ml of 0.03 M MnCl_2 solution was preincubated for 20 min at 40°C , and then 1 ml of 0.03 M D-xylose solution in 0.3 M borate buffer (pH 7.0) was added to this mixture. After incubation at 40°C for 30 min, the reaction was terminated by adding 1 ml of 1.0 M perchloric acid. For the assay of D-ribose-isomerizing activity, the procedure was the same as the assay of D-glucose-isomerizing activity, except that 1 ml of 0.06 M D-ribose was used. The ketoses formed were determined by the cysteine-carbazole method and enzymatic activities were expressed as values of the optical density given in the determination method.

Chemicals. Chemicals for buffer, substrates and

metal salts were special grade and purchased from the Wako Pure Chemical Industries, Ltd. Buffers, unless otherwise indicated, were sodium barbital-sodium acetate-hydrochloric acid system and adjusted to the desired pH at 40°C . Buffers and solutions of substrates were prepared with deionized water, followed by extraction with 0.003% (w/v) dithizone in CCl_4 to remove trace metals, and were stored in polyethylene containers at $2\sim5^\circ\text{C}$.

Equilibrium dialysis. Dialysis membrane bag was prepared from a cellulose tubing (6.4 mm) of Visking Company by the treatment with boiling EDTA solution, followed by washing with distilled water. In each experiment, 4 ml of enzyme solution in the buffer (see below) was pipetted into the bag. The bag was placed in 6 ml of the buffer containing CoCl_2 , and gently shaken for 48 hr at 5°C . Barbital buffer (0.02 M) containing 0.1 M NaCl was used in all experiments. Amounts of Co^{2+} in the other solution were measured with an atomic absorption spectrophotometer (Hitachi, model 207).

RESULTS AND DISCUSSION

Effect of metal concentration on the activities

As shown in Fig. 1, the enzyme required

FIG. 1. Effect of Co^{2+} , Mn^{2+} and Mg^{2+} Concentration on the D-Glucose-(A), D-Ribose-(B) and D-Xylose-(C) Isomerizing Activities.

The reaction mixtures were the same as those described under "Materials and Methods," except that the indicated concentration of metal salt was used.

○, CoCl_2 ; ●, MnCl_2 ; △, MgCl_2 .

Co^{2+} for both D-glucose- and D-ribose-isomerizing activities. The optimal concentration of Co^{2+} was 0.01 M. At higher concentration than 0.01 M, Co^{2+} showed inhibitory effect. For D-xylose-isomerizing activity of the enzyme, on the other hand, Mn^{2+} was essential, and optimal concentration of Mn^{2+} was about 0.01 M. Although the maximum D-xylose-isomerizing activity was obtained at a concentration of 10^{-5} M of Co^{2+} , the activity is corresponded to approximately a quarter of the maximum activity given by Mn^{2+} (10^{-2} M). The activity with Co^{2+} (10^{-5} M) showed a similar value obtained at the same concentration of Mn^{2+} .

Effect of the addition order of the components to the reaction mixtures on the reaction rate

When metal ion activates the enzyme reaction, there are generally two possible cases,

i.e., metal ion can bind to a enzyme or/and substrate and form active enzyme or/and active substrate. Therefore, effect of preincubation of the enzyme or substrate with metal ion was investigated. As shown in Fig. 2, when the D-glucose-isomerizing activity was assayed without preincubation with Co^{2+} , the initial reaction velocity was very low and increased with time. After the preincubation with Co^{2+} for 4 hr at 20°C , the reaction started linially from zero time. When the enzyme was added to the mixture in which D-glucose was preincubated with Co^{2+} for 4 hr at 20°C , the reaction velocity was little. The similar results were also obtained on D-xylose-isomerizing system composed of the enzyme, Mn^{2+} and D-xylose. These results suggested that the primary reaction in the activation process was a binding of metal ion to the enzyme protein, so that Co^{2+} - and Mn^{2+} -enzyme complexes were active form of the enzyme.

FIG. 2. Effect of the Adding Order of the Components in the Reaction Mixture on the Rate of D-Glucose (A) and D-Xylose (B) Isomerization.

Reaction systems: (A) 0.01 M CoCl_2 , 0.2 M D-glucose, 50 μg enzyme, (B) 0.01 M MnCl_2 , 0.01 M D-xylose, 4 μg enzyme, 0.007 M barbital buffer (pH 7.0), total volume, 5 ml.

○, mixture of enzyme and metal was incubated at 20°C for 4 hr and then added substrate; △, substrate and metal was incubated at 20°C for 4 hr and then added enzyme; ●, enzyme metal and substrate were mixed simultaneously.

Effect of pH on the rate of activation of the enzyme by Co^{2+} and Mn^{2+}

Activation rate was determined after the preincubation for 10 min at 20°C at various pHs given by barbital buffer and expressed

FIG. 3. Effect of pH on the Rate of Activation of the Enzyme with Co^{2+} and Mn^{2+} .

Experimental condition was described in the text. A, D-glucose-isomerizing activity (Co^{2+}), B, D-xylose-isomerizing activity (Mn^{2+}).

as relative activity. As shown in Fig. 3, the activation rate was markedly pH-dependent, *i.e.*, the activation by the metal ion was most rapid at pH 6.0, and was slow at both side

FIG. 4. Effect of Temperature on the Rate of Activation of the Enzyme with Co^{2+} .

The enzyme solutions ($50 \mu\text{g}$) in barbital buffer were pre-incubated at indicated temperature with CoCl_2 (0.01 M). After incubation for 10 min, the solution was cooled immediately in an ice bath, and then D-glucose was added and activity was measured.

Δ , pH 6.7; $\circ$, pH 7.0; $\bullet$, pH 7.5.

FIG. 5. Effect of pH on the Binding of Co^{2+} to the Enzyme under Fixed Concentration of Co^{2+} .

Number of Co^{2+} bound to the enzyme was determined by equilibrium dialysis as described in "Materials and Methods."

of the pH. During the 10-min preincubation at pH 7.0, the activity reached only 40~50% levels of the maximum activity. The effect of temperature during 10-min incubation with Co^{2+} on activation rate was measured. As shown in Fig. 4, the activation rate which induced by preincubation increased with a rise of temperature. Molar ratio of Co^{2+} bound to the enzyme under fixed concentration of Co^{2+} increased with pH (Fig. 5). From above data, it was considered that the enzyme molecule forms a structure which metal ion is rapidly bound to the enzyme at pH 6.0.

Kinetics of activation by metal ions

When metal ion forms an essential part in active center of the enzyme, there are two possible cases. First, it is assumed that metal ion is located in the binding site of the active center of the enzyme, where metal ion acts as a connecting point of the substrate as shown for carboxypeptidase³⁾ and D-xylose ketol-isomerase from *Lactobacillus brevis*.⁴⁾ If only the enzyme-metal ion complex is able to combine with substrate, following reaction mechanism may be considered

The equation for the velocity is as follows:

$$v = \frac{ke}{1 + \frac{Km}{s} \left(1 + \frac{Ka}{a} \right)} \quad (2)$$

where a , s and e are the concentrations of metal ion, substrate and enzyme, respectively, and K_m represents the Michaelis constant and K_a is the dissociation constant of the enzyme-metal complex. For testing the applicability of equation (2) to the experimental data, it is conveniently written in a form suggested

3) M. Dixon and E. C. Webb, "Enzyme," Academic Press Inc., N. Y., 1964.

4) K. Yamanaka, *Mem. Fac. Agr. Kagawa Univ.*, **16**, (1963).

by Lineweaver and Burk:

$$\frac{1}{v} = \left(1 + \frac{Ka}{a}\right) \frac{1}{V} \frac{Km}{s} + \frac{1}{V} \quad (3)$$

Second, the metal ion acts as stimulant of the catalytic function of the enzyme, as shown for beta-methylasparatase.⁵⁾ If the binding of substrate to the enzyme is not subjected to the metal ion, following reactions may be considered

The equation for the velocity is as follows:

$$v = \frac{ke}{\left(1 + \frac{Ka}{a}\right) \frac{Km}{s} + \left(\frac{Ka}{a} \cdot \frac{Km}{Ks} + 1\right)} \quad (5)$$

where a , s and e are the concentrations of metal ion, substrate and enzyme, respectively,

FIG. 6. Double Reciprocal Plots of the Reaction Rate and Concentration of D-Glucose at the Indicated Concentration of Co^{2+} .

Experimental condition was the same as described in "Materials and Methods," except that indicated concentration of Co^{2+} and D-glucose were used. Concentration of CoCl_2 : $\circ$, 10^{-3} ; $\bullet$, 5×10^{-4} ; $\triangle$, 2.5×10^{-4} ; $\blacktriangle$, 1.25×10^{-4} ; $\square$, 6.3×10^{-5} M.

FIG. 7. Double Reciprocal Plots of the Reaction Rate and Concentration of D-Glucose at the Indicated Concentration of Mn^{2+} .

Experimental condition was the same as described in "Materials and Methods," except that indicated concentration of Mn^{2+} and D-glucose were used. Concentration of MnCl_2 : $\circ$, 10^{-3} ; $\bullet$, 5×10^{-4} ; $\triangle$, 2.5×10^{-4} ; $\blacktriangle$, 1.25×10^{-4} M.

FIG. 8. Double Reciprocal Plots of the Reaction Rate and D-Xylose Concentrations at the Indicated Concentrations of Mn^{2+} .

Experimental condition was the same as described in "Materials and Methods," except that indicated concentration of metal and D-xylose were used. Concentration of MnCl_2 : $\circ$, 10^{-3} ; $\bullet$, 5×10^{-4} ; $\triangle$, 2.5×10^{-4} ; $\blacktriangle$, 1.25×10^{-4} ; $\square$, 6.3×10^{-5} M.

5) H.J. Bright, *J. Biol. Chem.*, **240**, 1198 (1965).

and K_m represents the Michaelis constant and K_a is the dissociation constant of the enzyme-metal complex. K_s is replaced by K_m and then equation (5) is reformed as follows:

$$\frac{1}{v} = \frac{K_m}{V} \left(1 + \frac{a}{K_a}\right) \frac{1}{s} + \frac{1}{V} \left(1 + \frac{a}{K_a}\right) \quad (6)$$

In order to clarify which mechanism is more appropriate as a function of metal for isomerizing reaction in the D-glucose-isomerizing enzyme, the following experiments were performed. The reaction rates of D-glucose isomerization at the varying level of divalent cations, such as Co^{2+} and Mn^{2+} , were plotted in double reciprocal forms. As shown in Figs. 6 and 7, the lines obtained at each concentration levels of Co^{2+} gave the same intercepts on abscissa, this results indicated that K_m value was not affected by the concentration of the cations. As shown in Fig. 8, a similar results were obtained for D-xylose-isomerizing reaction. Since above data clearly showed that the values of the intersection on the abscissa remained unchanged when concentrations of metal ion was varied, equation (6) is fit for this data. Thus, it was suggested that the function of Co^{2+} and Mn^{2+} in the D-glucose- and D-xylose-isomerizing reactions might be a stimulation of the catalytic activity on the catalytic site of the enzyme.

Equation (6) is reformed as a function of metal concentration

$$\frac{1}{v} = \left(1 + \frac{K_m}{s}\right) \frac{K_a}{V} \cdot \frac{1}{a} + \left(1 + \frac{K_m}{s}\right) \frac{1}{V} \cdot \quad (7)$$

Equation (7) predicts that the intersection of straight lines occurs on the abscissa in double reciprocal plot of reaction rate against metal ion concentration at different substrate concentrations. The numerical value of $1/a$ at the point of intersection of lines in the plot of $1/v$ versus $1/a$ indicates the value of $-1/K_a$. The data in Figs. 6 and 8 are replotted in Fig. 9 and 10, respectively, as a function of Co^{2+} and Mn^{2+} at a constant concentration of the substrate. Dissociation constants (K_a)

of enzyme- Co^{2+} and enzyme- Mn^{2+} was calculated to be $1.8 \times 10^{-4} \text{ M}$ and $9 \times 10^{-5} \text{ M}$, respectively. This value is in good agreement with the value of $4.5 \times 10^{-5} \text{ M}$ obtained from the equilibrium dialysis experiment which will be described below. That is, the dis-

FIG. 9. Double Reciprocal Plots of the Reaction Rate and Co^{2+} Concentrations at the Indicated Concentrations of D-Glucose.

Data of Fig. 6 were replotted. D-Glucose concentration: ○, 0.2; ●, 0.1; △, 0.05; ▲, 0.033; □, 0.025 M.

FIG. 10. Double Reciprocal Plots of the Reaction Rate and Mn^{2+} Concentrations at the Indicated Concentrations of D-Xylose.

Data of Fig. 8 were replotted. Concentrations of D-xylose: ○, 0.01; ●, 0.005; △, 0.0033; ▲, 0.0025 M.

FIG. 11. Binding of Co^{2+} by the Enzyme at pH 7.0.

r , the number of molecules of Co^{2+} bound per molecule of protein was determined by the equilibrium dialysis method as described in "Materials and Methods."

sociation constant (Ka) by the equilibrium dialysis method was calculated from the following equation (6)

$$r \frac{Ka}{a} = n - r \quad (8)$$

where r is the average number of Co^{2+} bound per molecule of the enzyme, a is concentration of free Co^{2+} , and n is the total number of binding sites in the enzyme. A plot of r versus r/a from the results of several Co^{2+} -binding experiments is shown in Fig. 11. The line had the parameters of $n=4$ and $Ka=4.5 \times 10^{-5} \text{ M}$.

Effect of Co^{2+} and Mn^{2+} on nonenzymatic isomerization

Effects of Co^{2+} and Mn^{2+} for nonenzymatic isomerization of the sugars were investigated. Solutions containing D-glucose or D-xylose, metal salt, and barbital buffer were adjusted to pH 6.8 with 0.5M NaOH and were incubated at 50°C in screw-capped test tubes. At various time intervals, aliquots were re-

FIG. 12. Effect of Metal Ion on the Nonenzymatic Isomerization of D-Glucose (A) and D-Xylose (B).

D-Glucose or D-xylose (1 m mole) was incubated at 50°C with metal salt (1 m mole in chloride form) in a final volume of 10 ml. The pH of the incubation mixtures was 6.8, and barbital buffer (0.007 M) was employed. At the indicated intervals, 0.2 ml aliquots were withdrawn from the incubation mixture and diluted with water and ketoses formed were determined by cysteine-carbazole method: ■, no addition; ○, Ni^{2+} ; ●, Co^{2+} ; ●, Mn^{2+} ; △, Mg^{2+} ; ▲, Na^{+} ; □, EDTA.

moved from the incubation mixtures and diluted with water, and ketoses formed were determined by cysteine-carbazole method. As shown in Figs. 12 and 13, stimulative effects of metal salt on the nonenzymatic isomerization were observed with a slow rate. Among the metal salts used here, Ni^{2+} gave the highest effect on the isomerization of both D-glucose and D-xylose. Thus, a functional role of metal ion in the enzymatic reaction

6) J. E. Hayes and S. F. Velick, *J. Biol. Chem.*, **207**, 225 (1954).

FIG. 13. Nonenzymatic Isomerization of D-Glucose (A) and D-Xylose (B) as Function of Metal Salt Concentration.

Experimental condition was the same as described in Fig. 12, except that indicated concentration of metal salt was used and reaction time was 70 hr: $\odot$, NiCl_2 ; $\circ$, CoCl_2 ; $\bullet$, MnCl_2 .

may be thought to be an conformational change in enzyme molecule, rather than in stimulating effect for the isomerization.

Difference of the binding site of Co^{2+} and Mn^{2+}

It is of interest to determine whether the binding site of Co^{2+} and Mn^{2+} was the same or not. Mn^{2+} inhibited D-glucose-isomerizing activity by Co^{2+} -activated enzyme. Figure 14 is double reciprocal plot of the reaction rate as a function of Co^{2+} concentration under a fixed level of D-glucose and in the presence or absence of Mn^{2+} (10^{-4}M). The mode of inhibiting action by Mn^{2+} was found to be partially non-competitive. This results means that Co^{2+} and Mn^{2+} did not compete at the binding site. Furthermore, this is strongly supported from following experiment of inhibition of Ni^{2+} . Figures 15 and 16 are double reciprocal plots of the reaction rate as a function of Co^{2+} or Mn^{2+} concentration under a fixed level of D-glucose or D-xylose, and in the presence or absence of Ni^{2+} (10^{-4}M). On the D-glucose-isomerizing activity by Co^{2+} -activated enzyme, Ni^{2+} inhibited the activity in competitive type as shown in Fig. 15. On

FIG. 14. Double Reciprocal Plots of the Reaction Rate and Co^{2+} Concentration in the Presence or Absence of Mn^{2+} and at a Fixed Concentration of D-Glucose.

Experimental condition was the same as described in "Materials and Methods," except that indicated concentration of metal ion and D-glucose (0.3M) were used: $\bullet$, with MnCl_2 (10^{-4}M); $\circ$, without MnCl_2 .

the D-xylose-isomerizing activity by Mn^{2+} -activated enzyme, however, Ni^{2+} inhibited the activity in non-competitive type. Since

FIG. 15. Double Reciprocal Plots of the Reaction Rate and Co^{2+} Concentrations in the Presence or Absence of Ni^{2+} and at Fixed Concentration of D-Glucose (A) or D-Ribose (B).

Experimental condition was the same as described in "Materials and Methods," except that indicated concentrations of metal and substrate (D-glucose, 0.3 M; D-ribose, 0.2 M) were used: ●, with Ni^{2+} (10^{-4} M); ○, without Ni^{2+} .

FIG. 16. Double Reciprocal Plots of the Reaction Rate and Mn^{2+} Concentrations in the Presence or Absence of Ni^{2+} .

Experimental condition was the same as described in "Materials and Methods," except that indicated concentrations of metal ion and D-xylose (0.1 M) were used: ●, with Ni^{2+} (10^{-4} M); ○, without Ni^{2+} .

the inhibition of Ni^{2+} was competitive for Co^{2+} and non-competitive for Mn^{2+} , it is concluded that the binding site of Co^{2+} and Mn^{2+} on the enzyme is different from each other.

Mechanism proposed for activation by metal ions

On the basis of the results described above, a possible function of metal ions for the isomerizing reaction and for substrate specificity of the enzyme is proposed, and is illustrated in Fig. 17. The assumption has been made that on the active center the enzyme protein has three binding sites; binding site ①, ② and ③ for substrate, Co^{2+} and Mn^{2+} , respectively. On the site ①, D-glucose (or D-ribose) or D-xylose may bind to the enzyme with an

FIG. 17. Proposed Mechanism for Activation of the Enzyme with Co^{2+} and Mn^{2+} .

Details are described in the text.

affinity which is not affected by metal ions. When Co^{2+} binds site ②, the conformational change in the enzyme molecule may occur to give an active form (form A) showing high activity for D-glucose isomerization. When Mn^{2+} bind to site ③, another active form (form B) having high activity for D-xylose isomerization is induced.

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