Kinetic and Equilibrium Studies on D-Mannose-D-Fructose Isomerization Catalyzed by Mannose Isomerase from *Streptomyces aerocolorigenes*

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The equilibrium constant of the isomerization reaction between D-mannose and D-fructose which is catalyzed by a mannose isomerase from *Streptomyces aerocolorigenes* was obtained by using three methods over the temperature range from 1 to 40°C.

It was found that the equilibrium constant was scarcely dependent on temperature, ΔH , the heat of the formation of D-fructose from D-mannose, being approximately zero.

The standard free energy change, ΔG , and the standard entropy change, ΔS , of the reaction were calculated from the equilibrium constants at various temperatures and ΔH . The values of ΔG and ΔS at 25°C were -650 cal/mole and +2.2 cal/deg·mole, respectively.

By combining these thermodynamic data with those obtained for the isomerization reaction between D-glucose and D-fructose reported in the previous paper, ΔH , ΔG and ΔS for the isomerization between D-mannose and D-glucose were indirectly obtained to be +2220 cal/mole, +830 cal/mole and +4.6 cal/deg·mole at 25°C, respectively.

INTRODUCTION

Mannose isomerase catalyzes the reversible interconversion between D-mannose and D-fructose.

In the previous paper,²⁾ the formation of the isomerase from *Streptomyces aerocolorigenes* and some of its properties were reported by the author.

This paper deals with the influence of temperature upon the equilibrium constant of the reaction catalyzed by this enzyme.

EXPERIMENTAL

Enzyme preparation. The cell-free extracts (100 ml), which were prepared according to the method shown in the previous paper,²⁾ were brought to 40% saturation by the addition of solid ammonium sulfate. After removal of the precipitate by centrifugation, the

supernatant was brought to 60% saturation by further addition of ammonium sulfate. The precipitate was collected and dissolved in distilled water, and then dialyzed in a cellophane tube against distilled water for 24 hours. The dialyzed sample was treated with 1.8 ml of 2% protamine and then centrifuged, and the supernatant (protein concentration, 5.6 mg/ml) was used as a stock enzyme solution throughout this experiment.

Materials. D-Mannose and D-fructose were purchased from Shimahisa Yakuhin Co. (Japan) and Wako Junyaku Co. (Japan), respectively. The purity of these sugars was checked by measuring their specific rotations at 589 m μ . These were in good agreement with their specific rotations reported in the literature.

Procedure. Enzyme reactions were carried out at the temperature range of 1 to 40°C in a 0.05 M phosphate buffer solution (pH 7.0). The time course of the reaction was followed by one of the following two methods.

1) **Chemical analysis method.** The reaction was commenced by mixing 1.5 ml of the stock enzyme

1) Y. Takasaki, This Journal, **31**, 309 (1967).

2) Y. Takasaki, *Report of Fer. Res. Inst.*, **28**, 89 (1965).

solution with 1.5 ml of substrate solution in 0.1 M phosphate buffer solution (pH 7.0) (concentration of substrate, D-mannose or D-fructose, 0.02%) in a test tube. At definite time intervals, 0.5 ml of the reaction mixture was pipetted out and was poured immediately into 0.5 ml of 0.5 M perchloric acid to stop the reaction. The concentration of D-fructose formed or remained was determined by the cysteine-carbazole method.³⁾

2) Polarimetric method. The reaction was commenced by mixing 0.1 ml of the stock enzyme solution with 1 ml of the buffer-substrate solution (substrate, D-mannose or D-fructose, 0.1~1.0% in 0.05 M phosphate buffer solution (pH 7.0)) in a quartz cuvette (optical path 10 mm) in a recording-spectropolarimeter (Yanagimoto ORD-185 type), and the change in optical rotation was recorded at 300 m μ as described in the preceding paper.¹⁾ When 1 mole of D-mannose is converted into 1 mole D-fructose, the decrease in optical rotation should be 8.98° on the basis of optical rotation was recorded at 300 m μ as described in the preceding paper.¹⁾ When 1 mole of D-mannose is converted into 1 mole D-fructose, the decrease in optical rotation should be 8.98° on the basis of optical rotation for D-mannose and D-fructose obtained at 300 m μ under these conditions. The maximum sensitivity of the polarimeter-recorder system used here was 0.005° (rotational angle) per 1.5 cm of the recorder chart.

RESULTS AND DISCUSSION

The equilibrium constant K of the isomerization for the formation of D-fructose from D-mannose is given by the expression

$$K = \frac{[\text{D-Fructose}]}{[\text{D-Mannose}]} \quad (1)$$

I. Determination of equilibrium constant

The equilibrium constant was determined by the following three methods, two of which were essentially the same as those described in the preceding paper.¹⁾ The third one, termed the equilibration rate method, was employed in addition in this paper.

1) Chemical analysis method. Fig. 1. shows a typical time course (studied by analytical method) of the mannose isomerase-catalyzed reaction which was carried out at 25°C using either pure D-mannose or pure D-fructose. The lower curve represents the time course

FIG. 1. Typical Time Course of Mannose Isomerase Reaction as Studied by Chemical Analysis Method

Reaction mixture

{ Initial substrate concentration, $10 \times 10^{-2}\%$ (for each sugar)
 { Phosphate buffer solution (pH 7.0), $5.0 \times 10^{-2}\text{M}$
 { Enzyme concentration, $2.8 \times 10^{-1}\%$ (as protein)
 Temperature, 25°C

○—○ D-Mannose (as starting substrate)
 ●—● D-Fructose (as starting substrate)

* Conversion Ratio; $\frac{[\text{D-Fructose}] \times 100}{[\text{D-Mannose}] + [\text{D-Fructose}]}$

FIG. 2. Plots of $\log K$ versus $1/T$

Reaction mixture

{ Substrate concentration, $1.0 \times 10^{-2}\%$
 { Enzyme concentration, $2.8 \times 10^{-1}\%$ (as protein)
 Temperature range, 1~40°C

of formation of D-fructose starting from pure D-mannose and the upper curve shows the time course of decrease in D-fructose starting from pure D-fructose. From the concentrations of D-fructose and D-mannose at equilibrium state, the equilibrium constants were determined in such a manner as shown in the preceding paper. As a result, the equilibrium constant K was estimated to be 3.0.

Fig. 2 shows a plot of logarithms of the equilibrium constants determined at several

3) Z. Dische and E. Borenfreund, *J. Biol. Chem.*, **192**, 583 (1951).

temperatures against reciprocals of absolute temperature.

It was found that the equilibrium constant of the D-mannose-D-fructose system is scarcely dependent on temperature. Consequently, the heat of the reaction ΔH is evaluated to be zero within the experimental error.

2) **Kinetic method.** The equilibrium constant can also be determined from the maximum velocities (V) and the Michaelis constants (K_m) of both forward and backward reactions according to Haldane's equation as described in the preceding paper.

The plot of s/v versus s , where v is the initial velocity at the substrate concentration s , is shown in Fig. 3.

$$\frac{s}{v} = \frac{K_m}{V} + \frac{s}{V} \quad (2)$$

From the intercepts on the base line of these plots, the values of K_m for D-mannose and D-fructose were calculated to be $1.4 \times 10^{-3}M$ (0.26%) and $2.1 \times 10^{-3}M$ (0.38%), respectively. Since the intercept on the vertical axis gives

FIG. 3. Plots of s/v versus s

Enzyme concentration, $5.1 \times 10^{-2}\%$ (as protein)

Temperature, 25°C

●—● D-Mannose

○—○ D-Fructose

TABLE I. KINETIC PARAMETERS AND EQUILIBRIUM CONSTANT OF MANNOSE ISOMERASE REACTION

Temperature (°C)	K	K_{mf} (%)	K_{mb} (%)	V_f (milli deg/min)	V_b (milli deg/min)	V_f/K_{mf}	V_b/K_{mb}
25	2.6	0.26	0.38	0.071	0.041	0.28	0.11

K_m/V , the equilibrium constant at 25° and pH 7.0 was estimated to be 2.6 according to Haldane's equation (equation (5) in the preceding paper¹). This value is in reasonable agreement with the value (3.0) at 25°C which was obtained by the chemical analysis method.

The values of K_m , V , V/K_m for the forward and backward reactions and K obtained by the method are summarized in Table I.

3) **Equilibration rate method.** This method is a new one devised to determine the equilibrium constant by the application of rate equation which was derived by Peller and Alberty.⁴

Consider a reaction involving the reversible interconversion of substrates A and B catalyzed by an enzyme E, in which two intermediate complexes EA and EB are assumed to be involved,

where k_{+1} , k_{+2} and k_{+3} are the rate constants for the forward processes and k_{-1} , k_{-2} and k_{-3} are the rate constants for the reverse processes, respectively.

The rate of disappearance of A, $-d[A]/dt$, or the rate of formation of B, $d[B]/dt$ (represented by v) is given by the steady-state treatment as follows:

$$v \equiv -\frac{d[A]}{dt} = \frac{d[B]}{dt} = \frac{\frac{V_A}{K_A}[A] - \frac{V_B}{K_B}[B]}{1 + \frac{[A]}{K_A} + \frac{[B]}{K_B}} \quad (4)$$

where $[A]$ and $[B]$ are the concentration of A and B at which the rate v is measured, V_A (or V_B) and K_A (or K_B) are the maximum

4) L. Peller and R. A. Alberty, *J. Am. Chem. Soc.*, **81**, 5907 (1959).

velocity and the Michaelis constant which are obtained by the usual method starting from the pure substrate A (or B), respectively.

The explicit expression for K_A , K_B , V_A and V_B in terms of k 's, which would be obtained from the generalized equation given by Peller and Alberty, is not necessary for the present purpose.

By using the Haldane relationship

$$K = \left(\frac{V_A}{K_A} \right) / \left(\frac{V_B}{K_B} \right) = R_A / R_B \quad (5)$$

where $R_A = V_A/K_A$, $R_B = V_B/K_B$, Equation (4) becomes

$$v = \frac{R_A([A] - [B]/K)}{1 + \frac{[A]}{K_A} + \frac{[B]}{K_B}} \quad (6)$$

Let Δ be the deviation of the substrate concentration $[A]$ and $[B]$ from their equilibrium values $[A]_e$ and $[B]_e$, then

$$\begin{aligned} [A] &= [A]_e + \Delta \\ [B] &= [B]_e - \Delta \quad (\Delta \geq 0) \\ [A] + [B] &= [A]_e + [B]_e = [C]_{\text{total}} \end{aligned} \quad (7)$$

where $[C]_{\text{total}}$ is the sum of the substrate concentrations of A and B. Equation (6) can be rewritten by the use of equation (7) as follows:

$$v = \frac{[R]_A [C]_{\text{total}} \{(1+K)f_A - 1\} / K}{\left(1 + \frac{[A]_e}{K} + \frac{[B]_e}{K} \right) + \Delta \left(\frac{1}{K_A} - \frac{1}{K_B} \right)} \quad (8)$$

where f_A is the mole fraction of A.

$$f_A = \frac{[A]}{[A] + [B]} = \frac{[A]}{[C]_{\text{total}}} \quad (9)$$

If the second term of the denominator of equation (8) can be neglected with the first term, i. e.,

$$|\Delta| \ll [A]_e, [B]_e \quad \text{or} \quad K_A \approx K_B \quad (10)$$

equation (8) reduces to

$$v = \frac{[R]_A [C]_{\text{total}} \{(1+K)f_A - 1\}}{K \left(1 + \frac{[A]_e}{K_A} + \frac{[B]_e}{K_B} \right)} \quad (11)$$

Thus for a constant $[C]_{\text{total}}$, v is a linear function of f_A , so far as the conditions given by equation (10) is fulfilled; i. e., generally using $[A]$ and $[B]$ near the equilibrium values $[A]_e$ and $[B]_e$ ($|\Delta| \ll [A]_e, [B]_e$)*

The equilibrium constant K can be obtained from f_A , for which v becomes zero. Thus,

$$\begin{aligned} (1+K)f_A - 1 &= 0 \quad (\text{for } v=0) \\ K &= \frac{1}{f_A} - 1 \end{aligned} \quad (12)$$

Practically, this value of f_A may be obtained by plotting v against various values of f_A (near equilibrium as possible). The point of intersection of the straight line of the plot with the f_A axis will give the value of f_A for $v=0$.

This provides a useful means for determining K in a great accuracy. The accuracy is the best among the three methods employed in this study. The closeness of the values of K_A and K_B in this case (K_m 's of D-mannose and D-fructose described above) provides the advantage to use relatively wide range of Δ .

FIG. 4. Effect of Initial Mole Fraction of D-Mannose on Rate Velocity

Reaction mixture

- Total substrate concentration $[C]_{\text{total}}$, 1.82%
- Phosphate buffer solution (pH 7.0), $4.5 \times 10^{-2} \text{M}$
- Enzyme concentration, $5.1 \times 10^{-2} \%$
- Temperature, 39.4°C

* Equation (11) can easily be written also in the form, for which $v \propto \Delta$ for small Δ ; v is proportional to the deviation from equilibrium and becomes zero at equilibrium.

FIG. 5. Effect of Initial Mole Fraction of D-Mannose on Rate Velocity

Reaction mixture

Total substrate concentration $[C]_{\text{total}}$, 1.82%
 Phosphate buffer solution (pH 7.0), $4.5 \times 10^{-2} \text{M}$
 Enzyme concentration, $5.1 \times 10^{-2} \%$
 Temperature, 25°C

This results of this method are shown in Figs. 4 and 5, in which the initial rate v determined polarimetrically is plotted against the mole fraction of D-mannose of the starting mixed substrate solution.

The total concentration $[C]_{\text{total}}$ was fixed at 1.82%. The values of K thus determined were 3.00 and 3.03 at 39.4°C and 25°C, respectively, which are in good agreement with those obtained by the chemical analysis method.

II. Thermodynamic quantities of isomerization of D-mannose to D-fructose

The standard free energy change accompanying the reaction in which D-fructose is formed from D-mannose was calculated from the K values. Since ΔH of the reaction is zero, the standard entropy change ΔS of the reaction can be given by using the following equation (13)

$$\Delta S = \frac{\Delta H - \Delta G}{T} = -\frac{\Delta G}{T} \quad (13)$$

The thermodynamic quantities obtained at various temperatures are listed in Table II.

In the preceding paper,¹ the thermodynamic quantities accompanying the process in which D-fructose is formed from D-glucose were de-

TABLE II. THERMODYNAMIC QUANTITIES OF ISOMERIZATION OF D-MANNOSE TO D-FRUCTOSE AT VARIOUS TEMPERATURES

Temperature (°C)	K	ΔH (cal/mol)	ΔG (cal/mol)	ΔS (cal/deg. mole)
1	3.1	0	-610	+2.2
6	3.1	0	-630	+2.2
11	3.0	0	-620	+2.2
25	3.0	0	-650	+2.2
35	2.9	0	-650	+2.1
40	3.0	0	-680	+2.2

TABLE III. THERMODYNAMIC QUANTITIES OF ISOMERIZATION OF D-GLUCOSE TO D-MANNOSE AT 25°C

Temperature (°C)	K	ΔH (cal/mole)	ΔG (cal/mole)	ΔS (cal/deg. mole)
25	0.24	+2220	+830	+4.6

termined by the use of glucose isomerase from *Streptomyces* sp. By combining these values and the data given in Table II, the thermodynamic quantities of the reaction in which D-mannose is formed from D-glucose, which cannot be studied directly due to the lack of enzyme specifically catalyzing the reaction, can be calculated. The results are listed in Table III.

It should be mentioned that the terms, D-glucose, D-mannose and D-fructose, used in this study and the preceding paper, represent the equilibrium mixture of anomers (α and β) of each sugar, the interconversion equilibrium of which had been actually established in aqueous solution before the measurement was commenced. The reaction of enzyme-catalyzed isomerization between the sugars was conducted in such a low rate compared with that of the α - β anomerization that the rate-limiting step is the isomerization itself. The initial and final states implied in the description of thermodynamic quantities correspond, therefore, the equilibrium mixture of α - and β -anomers of each sugar.

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