Effects of Xanthine Oxidase on Synthesis of 5-Methyluridine by the Ribosyl Transfer Reaction

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In the presence of xanthine oxidase, 5-methyluridine was synthesized from inosine and thymine by the ribosyl transfer reaction using purine nucleoside phosphorylase and pyrimidine nucleoside phosphorylase. The hypoxanthine formed was converted into urate *via* xanthine by xanthine oxidase. After inosine was completely consumed, an equilibrium state, in which 5-methyluridine, thymine, ribose-1-phosphate, and phosphate were involved, was achieved. At the equilibrium state, maximum yield of 5-methyluridine was obtained. The equilibrium constant of the reaction ($K = [\text{thymine}][\text{ribose-1-phosphate}] / [\text{5-methyluridine}][\text{phosphate}]$) was 0.062. Based on the equilibrium constant, the yield of 5-methyluridine depended on a ratio of initial concentrations of inosine and phosphate, on condition that the initial concentrations of inosine and thymine were the same. The yields of 5-methyluridine in the presence and absence of xanthine oxidase were 76 and 33%, respectively, when the initial concentrations of inosine, thymine, and phosphate were 5 mM each.

Thymidine is produced in practice by hydrolysis of and separation from deoxyribonucleic acids derived from natural sources, such as milt. We developed a method for the practical production of thymidine from non-natural sources. 5-Methyluridine (ribosylthymine) (5MU) was synthesized from thymine (5-methyluracil) and inosine by the ribosyl-transfer reaction, using thermostable purine nucleoside phosphorylase (EC 2.4.2.1) (PUNP) and thermostable pyrimidine nucleoside phosphorylase (EC 2.4.2.2) (PYNP), as shown in the following chemical equations.¹⁻⁴⁾ Then, the hydroxy group at the 2'-position of 5MU was reduced chemically to yield thymidine.⁵⁾

The total reaction was as follows:

Although the efficient synthesis of 5MU was

crucial to produce thymidine in practice, the low yield of 5MU by this ribosyl transfer reaction was disadvantageous due to the low equilibrium constant of this reaction. As the equilibrium constant of the reaction is 0.24 at 60°C, the maximum yield of 5MU is 33% at the equilibrium state, on condition that initial concentrations of inosine and thymine are the same.⁶⁾ The yield does not depend on the concentration of phosphate.

We thought that if the ribosyl transfer reaction, *i.e.*, Eq. (3) was done in the presence of xanthine oxidase (EC 1.1.3.22) (XOD), the hypoxanthine formed would be converted into urate *via* xanthine by XOD. Since urate is not a substrate of PUNP or PYNP, the urate formed would not be involved in the ribosyl transfer reaction. As a result, the ribosyl transfer reaction would proceed to synthesize more 5MU, and a higher yield of 5MU would be obtained.

This paper describes the synthesis of 5MU in the presence of XOD by the ribosyl transfer reaction using PUNP and PYNP. It was shown that the yield of 5MU depended on a ratio of initial concentrations of inosine and phosphate

on condition that the initial concentrations of inosine and thymine were the same, and further that the yield of 5MU was 76%, when the initial concentrations of inosine, thymine, and phosphate were 5 mM each.

Materials and Methods

Microorganism. *Bacillus stearothermophilus* JTS 859, which produces two kinds of thermostable PUNPs and one kind of thermostable PYNP, was used as the enzyme source. Culture conditions of this strain were described in a previous paper.²⁾

Chemicals. Thymine was purchased from Wako Pure Chemical Industry (Tokyo). Inosine and 5MU were purchased from Yamasa Shoyu Co., Ltd. (Tokyo). All other reagents of analytical grade were commercially available.

Xanthine oxidase derived from cow milk was purchased from Boehringer Mannheim (Mannheim, Germany). One unit of XOD activity was defined as the amount of XOD that produced 1 μ mol of urate by the oxidation of 1 μ mol of xanthine in one min at 25°C and pH 8.5.

Preparation of the crude enzyme. One gram of wet cells was suspended in 10 ml of 20 mM potassium phosphate buffer (pH 7.0) and then disrupted with an ultrasonic oscillator for 10 min (20 kHz, 0–10°C). After centrifugation (10,000 $\times g$, 10 min, 4°C), the supernatant was obtained.

Eight ml of acetone (–20°C) was added to 10 ml of the supernatant. After standing for 15 min below 5°C, the mixture was centrifuged (10,000 $\times g$, 10 min, 4°C). Six ml of acetone (–20°C) was added to the supernatant. After standing for 15 min below 5°C, the mixture was centrifuged (10,000 $\times g$, 10 min, 4°C). The precipitate was dissolved in 5 ml of 20 mM potassium phosphate buffer (pH 7.0). It contained 11.4 mg/ml protein and was used as the crude enzyme.

One unit of enzyme activity was defined as the amount of enzymes that produced 1 μ mol of 5MU in one minute at 60°C and pH 7.0, as described in a previous paper.⁶⁾

Ribosyl transfer reaction in the presence of XOD. The standard reaction mixture consisting of 5 mM inosine, 5 mM thymine, 5 mM potassium phosphate, 1 U of XOD, and 0.27 U of the crude enzyme in a total volume of 1 ml (pH 7.0) was incubated in a micro test tube with gentle aeration at 40°C for 11 hr. Fifty μ l of the reaction mixture was transferred to 750 μ l of MeOH to stop the reaction.

Analysis. Quantitative analysis of the bases and the nucleosides was done by HPLC. The operating conditions were as follows: column, Chemcosorb RP18-10 ($\phi 4 \times 300$

mm) (Chemco Scientific Co., Ltd.); column temperature 38°C; solvent, water–MeOH=95:5 (v/v); flow rate, 0.8 ml/min; detector, UV monitor 254 nm. Retention times of urate, hypoxanthine, xanthine, thymine, inosine, and 5MU were 3.8, 6.5, 7.1, 8.6, 9.9, and 11.3 min, respectively.

Results and Discussion

Difference between the ribosyl transfer reaction in the presence and absence of xanthine oxidase

The effects of XOD on the synthesis of 5MU by the ribosyl transfer reaction were examined, as shown in Fig. 1. The reaction in the absence of XOD reached the equilibrium state after 2 hr of the reaction, as shown in Fig. 1A. As the concentration of 5MU was 1.45 mM at the equilibrium state, the yield of 5MU was 29%. The equilibrium constant of the reaction ($K = [5MU][\text{Hypoxanthine}]/[\text{Inosine}][\text{Thymine}]$) was 0.21 at 40°C.

On the other hand, inosine was consumed completely after 6 hr of the reaction in the presence of XOD, and the concentrations of hypoxanthine and xanthine were very low during the reaction, as shown in Fig. 1B. The concentration of urate increased with time. Further, concentrations of formed 5MU and of residual thymine were 3.8 and 1.2 mM, respectively, after 6 hr of reaction. The yield of 5MU was 76%.

After inosine was consumed, *i.e.*, after 6 hr of the reaction, the concentrations of 5MU and thymine were steady at least for 5 hr. These

Fig. 1. Difference between the Ribosyl Transfer Reaction with or without Xanthine Oxidase.

The reaction mixture containing 5 mM inosine, 5 mM thymine, 5 mM phosphate, 0.27 U crude enzyme, and 0 U XOD (A), or 1 U XOD (B) in a total volume of 1 ml was incubated at 40°C. ●, hypoxanthine; ○, inosine; ■, thymine; □, 5-methyluridine; △, xanthine; ▲, urate.

results suggested that 5MU and thymine were involved in an equilibrium state. As 5MU, thymine, phosphate, and PYNP existed in the reaction mixture, we assumed that the reaction involving thymine, 5MU, phosphate, and ribose-1-phosphate (RPI), reached an equilibrium state according to Eq. (2), although the concentration of RPI was not measured experimentally.

Equilibrium constant determination

The equilibrium constant of the reaction (2) ($K = [\text{RPI}][\text{Thymine}]/[\text{5MU}][\text{Phosphate}]$) was 0.062, based on the experimental results shown in Fig. 2. Based on the equilibrium constant, 0.062, the concentrations of thymine and 5MU at the equilibrium state of the ribosyl transfer reaction in the presence of XOD (Fig. 1B) could be calculated. Initial concentrations of inosine, thymine, and phosphate were 5 mM each. For convenience of calculation, we assumed that the equilibrium state in the reaction mixture was reached stepwise as follows, although the following steps would happen simultaneously. First, inosine and phosphate were converted into ribose-1-phosphate (RPI) (5 mM) and hypoxanthine (5 mM) by PUNP completely. Second, hypoxanthine was converted completely into urate

via xanthine by XOD. Third, RPI (5 mM) and thymine (5 mM) were converted into 5MU and phosphate by PYNP to reach the equilibrium state, according to Eq. (2). As the initial concentrations of inosine and thymine were the same, concentrations of RPI and thymine at the equilibrium state were the same. Based on the equilibrium constant, 0.062 (Eq. (4)), concentration of thymine, $[X]$, at the equilibrium state could be calculated as shown in the following Eq. (5).

$$\frac{[\text{RPI}][\text{Thymine}]}{[\text{5MU}][\text{Phosphate}]} = 0.062 \quad (4)$$

$$X \cdot X / (5 - X)(5 - X) = 0.062 \quad (5)$$

therefore,

$$X = [\text{Thymine}] = 0.997 \quad \text{and}$$

$$[\text{5MU}] = 5 - [\text{Thymine}] = 4.003$$

The concentrations of thymine and 5MU were 0.997 and 4.003 mM, respectively. Observed concentration of thymine and 5MU were 1.2 and 3.8 mM, respectively, as shown in Fig. 1B. These calculations explained the experimental results well.

These results suggested that the yield of 5MU depended on the initial concentrations of inosine and phosphate in the reaction mixture, on condition that the initial concentrations of inosine and thymine were the same.

Effects of the ratio of initial concentrations of inosine and phosphate on the yield of 5MU

Effects of the ratio of initial concentrations of inosine and phosphate on the yield of 5MU were examined, on condition that the initial concentrations of inosine and thymine were the same. The range of the ratio varied from 0.2 to 4.

Good agreement between the experimental and the calculated concentrations of 5MU was obtained, as shown in Fig. 3. Further, when reaction mixture containing 3 mM inosine, 3 mM thymine, 20 mM phosphate, 4 U XOD, and 1.08 U of the crude enzyme in a total volume of 2 ml, was incubated for 7 hr at 40°C, concentrations of thymine and 5MU formed in

Fig. 2. Equilibrium Constant Determination.

The reaction mixture containing 5 mM 5MU, 5 mM phosphate, and 0.27 U of the crude enzyme in a total volume of 1 ml was incubated at 40°C till the equilibrium state was observed. The residual 5-methyluridine and the formed thymine were measured with time, and the values of K ($K = [\text{Thymine}][\text{Ribose-1-phosphate}]/[\text{5-Methyluridine}][\text{Phosphate}]$) were calculated.

Fig. 3. Effects of the Initial Concentrations of Inosine and Phosphate on the Yield of 5MU.

The reaction mixture consisting of thymine (indicated), inosine (same concentration as thymine), 5 mM phosphate, 1 U XOD, and 0.27 U of the crude enzyme, was incubated at 40°C. When the concentrations of thymine were 10, 15, and 20 mM in the reaction mixture, 1, 3, and 3 U of XOD were added further, respectively. After inosine was consumed completely and an equilibrium state was achieved, the 5MU formed was measured (—●—). A broken line shows the calculated concentration of 5-methyluridine at the equilibrium state, based on Eq. (4) in the text.

the reaction mixture were 1.34 and 1.66 mM, respectively, after inosine was completely consumed, *i.e.*, after 2 hr of reaction, and their concentrations were steady for 5 hr (data not shown). Calculated concentrations of thymine and 5MU were 1.37 and 1.63 mM, respectively, based on the equation (4) ($X \cdot X / (3 - X)(20 - X) = 0.062$, $X = [\text{Thymine}]$). Good agreement between the experimental and the calculated concentrations of thymine and 5MU was obtained in this case, too.

These results showed that the equilibrium state of the ribosyl transfer reaction with XOD was governed by Eq. (2), not Eq. (3), after the inosine was completely consumed. Therefore, the yield of 5MU depended on the initial concentrations of inosine and phosphate, on condition that the initial concentrations of inosine and thymine were the same.

Effects of the ratio of the initial concentrations of inosine and phosphate on the yield of 5MU was examined based on Eq. (4), on condition that the initial concentrations of inosine and thymine were the same. When initial concentrations of inosine and thymine were 2.5, 5, 10, 20, and 40 mM each, and the initial concentration of phosphate was constant, 5 mM in the reaction mixture, calculated concentrations of 5MU at the equilibrium state were 1.81 (yield 72.4%), 4 (80%), 8.61 (86.1%), 18.1 (90.5%), and 37.6 mM (94%), respectively. Therefore, the yield of 5MU was increased with the increase of the ratio of the initial concentrations of inosine and phosphate. To obtain a high yield of 5MU, the ratio of the initial concentrations of inosine and phosphate should be high, on condition that the initial concentrations of inosine and thymine are the same.

The ribosyl transfer reaction has been used for the synthesis of several nucleoside analogues, but low yield of synthesized nucleoside analogues were disadvantageous due to the low equilibrium constant.^{7,8)} In general, the ribosyl transfer reaction in the presence of XOD could improve the yield of synthesized nucleoside analogues, whose sugar moiety is a ribose.

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