Importance of 3′-Hydroxyl Group of the Nucleosides for the Reactivity of Thymidine Phosphorylase from *Escherichia coli*

Akihiko Hatano,* Aiko Harano, and Masayuki Kirihara
Department of Materials and Life Science, Shizuoka Institute of Science and Technology, 2200-2 Toyosawa, Fukuroi 437-8555

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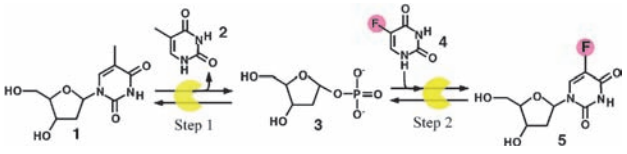
Thymidine phosphorylase in phosphate buffer catalyzed the conversion of thymidine to unnatural nucleosides. The 3′-OH, but not the 5′-OH of ribosyl moiety is necessary to be recognized as a substrate. Thus 3′-deoxythymidine could not convert to 5-fluorouracil-2′,3′-dideoxyribose. However, 5′-deoxythymidine was converted to 5-fluorouracil-2′,5′-dideoxyribose.

Unnatural nucleosides are of interest as antiviral and antitumor agents,[1] expansion of the genetic alphabet,[2–5] and functional building block molecules of DNA.[6,7] These molecules have been synthesized by a stereoselective coupling reaction of the base analogue and the protected ribose. These studies have a weak point because the coupling reactions of the base analogue and ribosyl moiety with Lewis acid produce mixtures of the 1′α and β unnatural nucleoside, and the yields are lowered.

Nucleoside phosphorylases[8–12] are intracellular metabolic enzymes that convert each of the corresponding four nucleosides to 2-deoxy-D-ribose-1α-phosphate. Thymidine phosphorylase (TP) catalyzed conversion of thymidine to thymine and 2-deoxy-D-ribose-1α-phosphate,[13,14] and it is known that TP is associated with tumor-dependent angiogenesis.[15–17] We expected that this enzyme could perform the transglycosylation of thymidine, because the enzymatic reaction was reversible. It is important to investigate the substrate specificities of this enzyme to assess if the process is a suitable synthetic method for development of unnatural nucleosides. Recently, the crystal structure of human TP was reported in a complex with uracil derivative.[18] This compound did not have a ribosyl moiety, and the ribose binding pocket was not defined since the iminopyrrolidine moiety had no OH groups. Schramm suggested a transition state of this enzyme that was a near-symmetric S_N2 nucleophilic displacement of thymine by inorganic phosphate.[19] They proposed that the 5′-OH of the ribosyl moiety may bind to the active-site residues by hydrogen bonding. In this communication, we studied the reactivities of thymidine phosphorylase (Thymidine: orthophosphate deoxyribosyltransferase, from *Escherichia coli*, EC 2.4.2.4), the recognized functional groups of the base moiety and the hydroxyl groups of the ribosyl moiety.

First, we looked for the most effective reaction conditions for each concentration of substrates (thymidine, base analogue, and phosphate ion). The reaction catalyzed by TP takes place in two steps (Scheme 1, step 1 and step 2) and these are reversible. Therefore, the produced thymine and the concentration of phosphate buffer inhibit step 2 under "one-pot" reaction conditions. Figure 1 shows the effects of concentration of substrates (thymidine (**1**), 5-fluorouracil (5FU, **4**)).[20] The conversion and initial velocity of the produced 5-fluorouracil-2′-deoxyribose (**5**) increased with increasing concentration of thymidine (the concentration of 5FU was constant at 5 mM). However, at a 10:1 ratio or higher of thymidine to 5FU the reaction leveled off. Figure 1b shows the effect of the 5FU concentration. As 5FU increased, the initial velocity decreased, and the conversion increased. The increasing concentration of 5FU shifted the



Scheme 1. Catalytic reaction of thymidine phosphorylase.

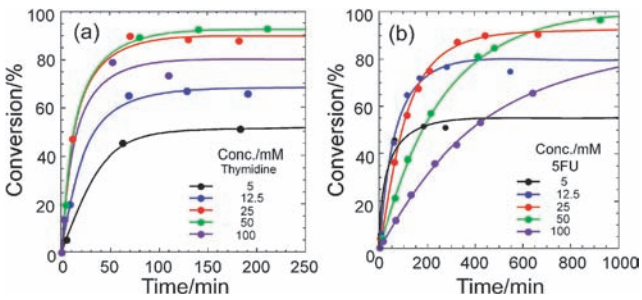
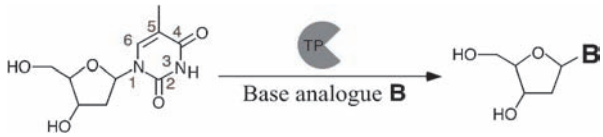


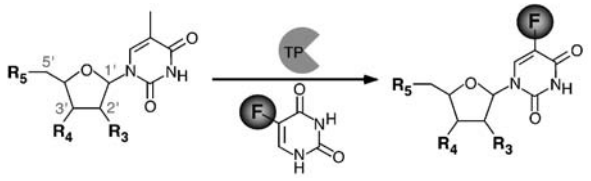
Figure 1. Effect of variation of substrate concentration on the conversion with thymidine phosphorylase. (a): Effect of changes in thymidine concentration. 5FU concentration was kept constant at 5 mM. (b): Effect of changes in 5FU concentration. Thymidine concentration was kept constant at 5 mM. All reactions were performed with 5 units of TP in 1 mM phosphate buffer (pH 7.0) at 35 °C.

Table 1. Effect of the base moiety on the thymidine phosphorylase reaction



Base analogue **B**	**R**	$\mathbf{R_1}$	$\mathbf{R_2}$	Time/h	Conv./%
6	H	O	O	2	95
4	F	O	O	2	86
7	Cl	O	O	2	87
8	Br	O	O	2	97
9	I	O	O	2	88
10	Et	O	O	2	67
11	NH_2	O	O	2	84
12	CF_3	O	O	2	47
13	NO_2	O	O	2	no reaction
14	H	O	S	2	54
15	CH_3	O	S	2	61
16	H	S	S	24	no reaction

Table 2. Effect of the hydroxyl group of the ribosyl moiety on the thymidine phosphorylase reaction



Substrate	R_3	R_4	R_5	Time/h	Conv./%
1	H	OH	OH	2	86
17	H	OH	**H**	3	85
18	H	**H**	OH	24	<1
19	H	**OH(β)**	OH	24	no reaction
20	H	**SH**	OH	24	<1
21	H	**NH_2**	OH	24	<1
22	**OH**	OH	OH	24	82

equilibrium and inhibited the production of 5-fluorouracil-2′-deoxyribose. The increasing concentration of phosphate ions inhibited the catalytic 5-fluorouracil conversion of thymidine to compound **5**. We decided to use the 10:1 ratio of thymidine (50 mM) to 5FU (5 mM) and 1 mM phosphate buffer (pH 7.0).[21]

Table 1 shows the substrate specificities of the base moiety in the TP-catalyzed reaction. Each of the 5-substituted uracil compounds (halogen: **4** and **7**–**9**, ethyl: **10**, amino: **11**, and trifluoromethyl: **12**) reacted with thymidine to convert the corresponding nucleoside in good yield. However, 5-nitrouracil **13** did not react with thymidine. 2-*S*-Substituted uracil (2-thiouracil **14** and 2-thiothymine **15**) in which oxygen at the 2 position of the base was replaced with sulfur reacted with thymidine to give 54 and 61%, respectively, of the corresponding nucleoside. Gago et al. predicted by molecular dynamic simulations and quantum chemical calculation that replacing the oxygen at position 2 of the pyrimidine base with sulfur should accelerate the reaction rate because of activation of the 2 position of uridine by His-85 of *E. coli* TP.[22] These authors studied the cleavage of the glycosidic bond of thymidine and 2-thiothymidine to base and 2-deoxy-D-ribose-1α-phosphate. We could not observe the acceleration by 2-thiothymine of the transfer reaction by TP. 2,4-Dithiouracil **16** did not dissolve in phosphate buffer, and the reaction did not occur.

However, not all pyrimidine base analogues were converted to the corresponded nucleosides. For example, the aza compounds, whose C-6 was replaced by a nitrogen atom, did not produce the nucleosides having a base analogue of an aza compound (azauracil and azathymine). Moreover, the compounds in which C-6 was substituted by methyl or keto groups could not be substrates (5,6-dimethyluracil, 5,6-dihydrouracil, and barbituric acid). We suggest that the C-6 position of the base substrate sterically hindered the nucleophilic attack to form an S_N2 transition state.

Table 2 shows the effect of the hydroxyl group of the ribosyl moiety on the catalytic reaction. To release the 5′-hydroxyl group, 5′-deoxythymidine **17** was reacted with 5-fluorouracil for 3 h to form 5′-deoxy-5-fluorouridine in a yield of 85%. This enzymatic reaction was nearly as effective as that of thymidine. Apparently, the 5′-OH group is not required to recognize the substrate for TP. In contrast to the 5′-OH reaction, the effect of 3′-OH was drastic. 3′-Deoxythymidine **18**, which does not possess the 3′-OH, did not react with 5FU to form 5-fluorouracil-2′,3′-dideoxyribose under the same conditions as above. Compounds **20** and **21**, in which the 3′-OH was replaced with SH and NH_2, respectively, showed a low conversion, and also 5FU did not react with 3′β-OH-thymidine **19**. These substrates most likely did not form hydrogen bonds with amino acids in the binding pocket of TP. Therefore, it can be concluded that the 3′α-hydroxyl group is very important for the recognition of the ribosyl substrate. Whereas the ribosyl 2′-OH retarded the catalytic reaction by TP, 5-fluorouridine was obtained in 82% yield from uridine **22**.

In conclusion, thymidine phosophorylase effectively catalyzed the reaction replacing the nucleobase of thymidine. We conclude that this enzyme recognized the 3′-OH of thymidine to fix the substrate.

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20 Typical procedure for reaction: To 10 mL of 0.1 M phosphate buffer (pH 7.0), were added thymidine (0.5 mmol, 121 mg), 5-fluorouracil (0.05 mmol, 6.5 mg), and thymidine phosphorylase (SIGMA, 5 units, from *Escherichia coli*, EC 2.4.2.4). The reaction mixture was stirred at 35 °C for 2 h. The reaction was monitored by HPLC.
21 Each conversion was assayed with a C-18 column (250–4.6 mm) HPLC at a flow rate of 0.5 mL min^{-1}. The mobile phase was 0.10 mM phosphate buffer (pH 6.8). The UV detector was set at 260 nm and the column was operated at 35 °C.
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