ENZYMATIC PYROPHOSPHORYLATION OF 2-AMINO-4-HYDROXY-6-HYDROXYMETHYL-7,8-DIHYDRO-PTERIDINE BY CELL-FREE EXTRACTS OF *ESCHERICHIA COLI* B

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Summary: Cell-free extracts of *Escherichia coli* B catalyze the direct pyrophosphorylation of ^{14}C-labelled 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine. Radioisotopic and chromatographic evidence show that the product enzymatically formed in this reaction is the pyrophosphate ester of the pteridine. ATP and Mg^{++} are essential as cofactors for the reaction. The pyrophosphorylation is strongly inhibited by 2-amino-4-hydroxy-6-carboxy-7,8-dihydropteridine.

In bacterial and plant enzyme systems[1)-3)] guanosine nucleotides can be converted into dihydropteroic or dihydrofolic acid. An early step in this transformation is removal of carbon 8 of GTP as formate[2),4)]. Studies on the effectiveness of various synthetic pteridines for the enzymatic formation of dihydropteroic acid[1),5)] have led to a proposal that the over-all pathway involves as intermediates, 2-amino-4-hydroxy-6-(D-*erythro*-1',2',3'-trihydroxypropyl)-7,8-dihydropteridine[2),3),6)], 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine[7)-9)] and the pyrophosphate ester of the latter pteridine[3),10)-12)]. These hypothetical compounds, however, had never been identified as enzymatic products in both plant and microbial systems. Recently, Jones and Brown[13)] obtained the propyldihydropteridine as a product from GTP in *E. coli* system, and demonstrated the conversion of this pteridine to the hydroxymethyldihydropteridine. In this communication, the evidence is provided for the direct enzymatic pyrophosphorylation of the hydroxymethyldihydropteridine.

EXPERIMENTAL

Materials: 2-Amino-4-hydroxy-6-carboxypteridine, Na salt[14)], 2-amino-4-

hydroxy-6-hydroxymethylpteridine[14], the mono- and pyrophosphate esters (Li salts) of the latter pteridine[15], and 2,4,5-triamino-6-hydroxypyrimidine sulfate-4-^{14}C (1 mC/m mole)[16] were prepared by the published methods. Microsynthesis of 2-amino-4-hydroxy-6-hydroxymethylpteridine-10-^{14}C (1 mC/m mole) was performed by condensation of 2,4,5-triamino-6-hydroxypyrimidine-4-^{14}C with dihydroxyacetone by the procedure of Rembold and Metzger[16] as used for the preparation of radioactive biopterin. The radioactive pteridine was purified by column chromatography on P-cellulose[16] and paper chromatography of *n*-butanol/acetic acid/H_2O (4:1:5) as a solvent system. The pteridines were reduced to the corresponding 7,8-dihydropteridines with sodium hydrosulfite for the enzyme reactions[17].

Cell-free extracts: The growth medium (pH 7.2) of *E. coli* B contained per liter: Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NH_4Cl, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 5 mg; glucose, 2 g; and $CaCl_2$, 33.3 mg. Each 2-liter flask containing one liter of the medium was inoculated with a 15 ml culture of the cell grown at 27° with shaking for 24 hours on the same medium. Growth of 8 liter cultures was allowed to proceed for 24 hours with continuous shaking at 27°. Cell pastes (28 g) harvested by centrifugation were ruptured by hand grinding with alumina (56 g) for 10 minutes in the cold. The mixture was suspended in 0.05 M potassium phosphate buffer (240 ml, pH 7.0). Grinding was continued for 5 minutes, then the suspension was centrifuged (10,000 r.p.m., 20 minutes, 0°). To the extract (215 ml), streptomycine sulfate solution was added to give a final concentration of 1.5 %. After stirring of the solution for 15 minutes at 4°, the precipitate was removed by centrifugation (10,000 r.p.m., 20 minutes, 0°). Solid ammonium sulfate was added to the supernatant fluid to give a 55 % saturated solution. Stirring was continued for another one hour at 4°. The protin precipitate recovered by centrifugation (10,000 r.p.m., 15 minutes, 0°) was dissolved in 0.05 M potassium phosphate buffer (15 ml, pH 7.0). The solution was dialyzed overnight at 4° against 5 mM potassium phosphate buffer (4 liters, pH 7.0). The dialysate, from which the resulting precipitate was

removed by centrifugation (10,000 r.p.m., 20 minutes, 0^{o}), was used as the enzyme preparation.

RESULTS

The radioactivity profile of the paper chromatogram of the reaction mixture showed only two peaks, as presented in Figure 1. These were coincident with the blue fluorescent bands observed under ultraviolet light. One peak corresponded definitely to 2-amino-4-hydroxy-6-hydroxymethylpteridine, the oxidized form of the substrate. The other was identical with the pyrophosphate ester of the hydroxymethylpteridine, but not with the monophosphate derivative.

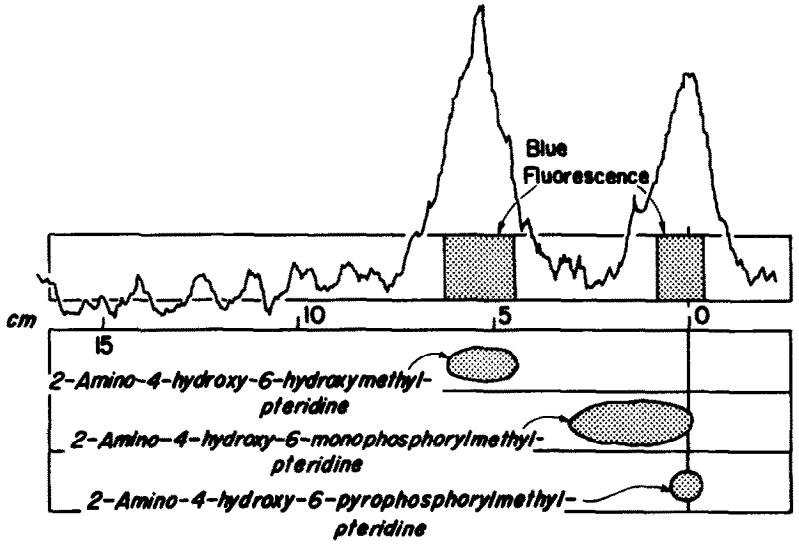

Figure 1. Radioactivity profile of the paper chromatogram of the reaction medium and chromatographic spots of the authentic pteridines. Reaction medium contained per 0.4 ml: ATP, 2.5 mM; $MgCl_2$, 5 mM; potassium phosphate (pH 7.0), 2.5 mM; 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine-10-^{14}C, 0.0613 mM (1 mC/m mole, 10,174 c.p.m. in a scintillation counting); sodium ascorbate, 1.8 mg; Tris-HCl buffer (pH 7.8), 100 mM; and 4.66 mg of enzyme protein. Incubation at 30^{o} was for 1.5 hour under air phase. After the reaction was stopped at the end of the period by immediate cooling and adding 0.1 ml of mercaptoethanol, 50 µl of the medium was streaked on a filter paper (Tôyô filter paper No. 51, 2.5 x 29.4 cm). Development of the chromatogram at room temperature was by the ascending method with a solvent of absolute ethanol/1 M ammonium acetate (pH 3.8)/1 M sodium EDTA (pH 8.2) (75:29:1). Authentic samples were chromatographed simultaneously using the papers previosly streaked on the respective start lines with 50 µl of the reaction media containing not the pteridine. Zones of migration were located by observing blue fluorescence under the ultraviolet light, or estimated with a paper radiochromatogram scanner.

Table I: Identification of 2-amino-4-hydroxy-6-hydroxymethylpteridine pyrophosphate ester by paper chromatography. At the end of the incubation for 2 hours, the reaction medium (see Figure 1) was immediately cooled and mixed with 5 mg of Norit A (acid-washed). After the suspension was stood at 4° for 10 minutes, the charcoal was collected by centrifugation (3,000 r.p.m., 5 minutes), and washed with cold distilled water (0.5 ml), followed by recentrifuging. This washing process was repeated further four times. Finally, the charcoal was suspended in 0.4 ml of absolute ethanol/3 N NH_4OH mixture (1:1). This mixture was allowed to stand at 4° for one hour with occasional shaking, and centrifuged. An aliquot (80 µl) of the supernatant fluid was applied with the authentic pteridines to the chromatography as the same technique described in Figure 1. Solvents used were: solvent 1, *n*-butanol/glacial acetic acid/H_2O (4:1:5); solvent 2, isoamyl alcohol/isopropanol/H_2O/*n*-butylic acid/28 % NH_4OH (75:25:75:120:2); solvent 3, absolute ethanol/1 M ammonium acetate (pH 3.8)/1 M sodium EDTA (pH 8.3) (75:29:1); solvent 4, 0.1 M potassium phosphate (pH 7.0); solvent 5, 4 % sodium citrate.

Solvents	R_f Values			
	Pt	Pt-P	Pt-PP	Enzymatic product
1	0.31	0.08	0.02	0.04
2	0.40	0.12	0.06	0.05
3	0.38	0.09	0.04	0.04
4	0.39	0.72	0.80	0.79
5	0.44	0.73	0.87	0.86

Pt, 2-amino-4-hydroxy-6-hydroxymethylpteridine; Pt-P, the monophosphate ester of this pteridine; Pt-PP, the pyrophosphate ester.

Table II: Requirements for Mg^{++} and ATP for enzymatic pyrophosphorylation of 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine. After incubated for 2 hours, the reaction medium (0.4 ml, see Figure 1) was chilled immediately in ice-water bath, followed by direct application of the medium on Dowex 1 x 2 (Cl^-) column (1.1 cm diameter, 1 ml volume). Residual substrate was first eluted with 50 ml of 0.02 M potassium acetate (pH 5.0). The pyrophosphate ester was obtained by elution with 5 ml of 0.5 M LiCl (pH 7.2). Radioactivity was determined in a Packerd scintillation spectrometer 314-EX.

Reaction system	c.p.m.
Complete	5,173
- ATP	580
- Mg^{++}	980
- Enzyme	767
- PtH_2	0
+ PtH_2-COONa (0.217 mM)	1,377

PtH_2, 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine; PtH_2-COONa, 2-amino-4-hydroxy-6-carboxy-7,8-dihydropteridine sodium salt.

The latter peak was absent, when Mg^{++} or ATP was omitted from the system. The enzymatic product was separated together with the residual substrate from the medium by charcoal adsorption, and further radiochromatographic analysis was

attempted. On every chromatogram developed with various solvents were detected only two blue fluorescent spots with radioactivity present; one agreed with the hydroxymethylpteridine and the other with its pyrophosphate ester. R_f values for the product and the authentic samples are shown in Table I. Incorporation of the radioactivity into the ester was examined after separating of the ester from the residual substrate. As shown in Table II, absence of either of Mg^{++} or ATP from the medium caused a marked decrease in the incorporation. The incorporation was extremely reduced when the oxidized substrate was used for the reaction. The reaction was also blocked by 2-amino-4-hydroxy-6-carboxy-7,8-dihydropteridine.

DISCUSSION

Cell-free systems of microorganisms[8),9)] and plants[7)] have been known to be able to catalyze the coupling reaction of 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine with *p*-aminobenzoic acid to yield dihydropteroic acid. Requirements for ATP and Mg^{++} in this reaction suggested a possible involvement of a phosphorylated intermediate. The final product can be formed enzymatically from the pyrophosphate ester of this pteridine without ATP, but not from the monophosphate ester even in the presence of Mg^{++} and ATP[3),10)-12)]. Pyrophosphorylation rather than successive phosphorylation of the substrate has been supposed a more plausible hypothesis, but no strict proof has been provided for this. The present work is the first demonstration of the pyrophosphorylating reaction, which requires ATP and Mg^{++} as cofactors. Although the reaction product isolated is characterized as the oxidized form, the pyrophosphate ester of the hydroxymethyldihydropteridine would be the immediate product of the reaction. This interpretation is supported by the following observations: (*a*) reducing power is not needed for the synthesis of dihydropteroic acid from the hydroxymethydihydropteridine or from its pyrophosphate ester[7)-9),12)], (*b*) the oxidized forms of these compounds do not act as the precursors[7)-9),12),18)], and (*c*) as confirmed in the present work the oxidized pteridine can not be pyrophosphorylated. The ester of the oxidized form is

obtained as a product only because the original dihydro compound is oxidized during the processes of chromatography and isolation. As shown in Figure 1, all the residual substrate is detected as the oxidized form on the chromatogram. Also, a single fluorescent and radioactive spot besides the oxidized substrate is confirmed on every chromatogram developed with the varied solvents. 2-Amino-4-hydroxy-6-carboxy-7,8-dihydropteridine inhibits the pyrophosphorylation, as indicated from the previous studies[5] on biosynthesis of dihydropteroic acid in higher plants.

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