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BIOSYNTHESIS OF RIBOFLAVIN

CHARACTERIZATION OF THE PRODUCT OF THE DEAMINASE

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The 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5′-phosphate deaminase was partially purified from cell extracts of *Candida guilliermondii* ATCC 9058. The enzyme requires Mg^{2+} for activity. Maximal activity was observed at pH 7.3. The enzyme converts its substrate, 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5′-phosphate, to 2,5-diamino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5′-phosphate. This labile compound was treated with diacetyl and the resulting 6,7-dimethyl-8-ribityllumazine 5′-phosphate was identified by comparison with a synthetic sample.

Introduction

The involvement of substituted pyrimidines in the biosynthesis of riboflavin was suggested on the basis of studies with riboflavin-deficient mutants of *Saccharomyces cerevisiae* [1–4]. The proposed sequence of events involved opening of the imidazole ring of the purine precursor, reduction of the ribose moiety and subsequent deamination of the pyrimidine ring at position 2 (Fig. 1). This hypothesis was based on the isolation of three pyrimidine compounds from the culture fluids of different riboflavin deficient mutants, namely 2,5,6-triamino-4(3H)-pyrimidinone [3], 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone [2] and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione [1]. However, attempts to demonstrate the expected enzymatic deamination of 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone were consistently unsuccessful, thus suggesting that the true biosynthetic intermediates might be phosphoric acid esters [5].

Positive evidence for the involvement of phosphoric acid esters was first obtained when Foor and Brown [6] isolated an enzyme, GTP cyclohydrolase, which converts GTP (I) to 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate (II). Burrows and Brown [7] obtained evidence for the further enzymatic conversion of the product of GTP cyclohydrolase. When ^{14}C-labeled II (prepared enzymatically from [^{14}C]GTP) was treated with two partially purified enzymes from *Escherichia coli* and subsequently with diacetyl and phosphatase, the authors were able to demonstrate the formation of ^{14}C-labeled 6,7-dimethyl-8-ribityllumazine (V). Evidence was obtained that deamination of the pyrimidine ring precedes the reduction of the ribose moiety and it was concluded that the pathway of riboflavin biosynthesis is different in bacteria and yeasts. Subsequent studies by Hollander and Brown [8] with the ascomycete *Ashbya gossypii* were in agreement with the pathway shown in Fig. 1.

The enzymatic conversion of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate reported

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'2-Amino-6,7-dimethyl-4-oxo-8-ribitylpteridine' should read as follows throughout the paper: '2-amino-6,7-dimethyl-4-oxo-8-ribityl-4,8-dihydropteridine'.

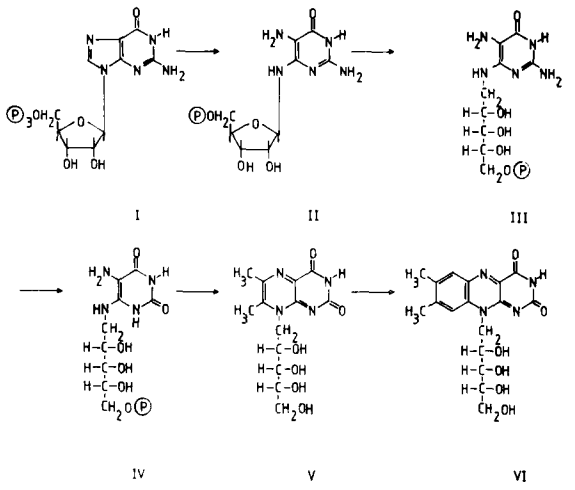

Fig. 1. Proposed pathway of riboflavin biosynthesis in yeast. I, GTP; II, 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate; III, 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5′-phosphate; IV, 5-amino-6-ribitylamino-2,4(1H, 3H)-pyrimidinedione 5′-phosphate; V, 6,7-dimethyl-8-ribityllumazine; VI, riboflavin.

by Burrows and Brown [7] was inhibited by the pretreatment of substrate with phosphatase. Hence, the authors concluded that the intermediates in the formation of 6,7-dimethyl-8-ribityllumazine should be phosphoric acid esters. However, these intermediates have not yet been isolated and characterized by direct chemical evidence.

We have recently observed an enzyme activity in *Candida guilliermondii* which deaminates synthetic 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5′-phosphate (III) in position 2 [9]. The enzyme activity was monitored by chemical conversion of the product to 6,7-dimethyl-8-ribityllumazine (V), which was subsequently converted to riboflavin by the enzyme riboflavin synthase. The riboflavin thus formed was measured by bioassay. Due to the presence of substantial phosphatase activity in the crude *C. guilliermondii* cell extracts we were unable to decide whether the deaminase produces 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione or the respective 5′-phosphate. We have now studied this question using a partially purified deaminase preparation from *C. guilliermondii*. The data show that the product of the enzyme is 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5′-phosphate (IV).

Materials and Methods

Chemicals. 2,5-Diamino-6-ribitylamino-4(3H)-pyrimidinone 5′-phosphate (III), 6,7-dimethyl-8-ribityllumazine 5′-phosphate and 2-amino-6,7-dimethyl-4-oxo-8-ribitylpteridine 5′-phosphate were synthesized (Bacher, A., unpublished data). 6,7-Dimethyl-8-ribityllumazine (V) and 2-amino-6,7-dimethyl-4-oxo-8-ribitylpteridine were prepared by published procedures [10,11]. Heavy riboflavin synthase was purified from *Bacillus subtilis* as described [12]. Alkaline phosphatase was purchased from Boehringer, Mannheim.

Organisms. C. guilliermondii ATCC 9058 was obtained from the American Type Culture Collection and was grown as described [13]. *Lactobacillus casei* ATCC 7469 was a gift of Dr. Bernhard Mailänder, Pfizer Inc.

Enzyme assays. Deaminase activity was determined as described [9]. One unit of deaminase activity catalyzes the formation of 1 nmol riboflavin per hour under the standard assay conditions.

Alkaline phosphatase activity was determined in glycine buffer, pH 10.5, with *p*-nitrophenylphosphate as substrate [14].

Miscellaneous methods. Protein was determined by the biuret method. Thin layer chromatography was performed with precoated Kieselgel 60 or Cellulose plates (Merck AG). Paper chromatography was performed with MN 218 paper (Macherey and Nagel, Düren); the solvent system was *n*-butanol/acetic acid/water (50 : 15 : 35, v/v). Electrophoresis was run on cellulose acetate strips (Macherey and Nagel, Düren) in 0.1 M imidazole/phosphate buffer pH 6.8 at 5.9 V/cm. Absorption spectra were measured with a Zeiss PM 6 spectrophotometer. The following extinction coefficients were used in calculations: 6,7-dimethyl-8-ribityllumazine, $\epsilon_{410} = 10\,300\ M^{-1} \cdot cm^{-1}$ at pH 2 [15]; 6,7-dimethyl-8-ribityllumazine 5′-phosphate, $\epsilon_{410} = 10\,300\ M^{-1} \cdot cm^{-1}$ at pH 2; 2-amino-6,7-dimethyl-4-oxo-8-ribitylpteridine, $\epsilon_{400} = 13\,200\ M^{-1} \cdot cm^{-1}$ at pH 2 [11]; 2-amino-6,7-dimethyl-4-oxo-8-ribitylpteridine 5′-phosphate $\epsilon_{400} = 13\,200\ M^{-1} \cdot cm^{-1}$ at pH 2.

Buffers. All buffers used contained 5 mM Mg^{2+}.

Preparation of cell extracts. Frozen cells were thawed and treated with glass beads in a Brown homogenizer for 14 min. The slurry was diluted with

0.1 M phosphate buffer pH 7.0 and filtered through a porous glass disc with suction. The filtrate was centrifuged and the pellet discarded.

Partial purification of deaminase. Cell extract of *C. guilliermondii* was brought to 31% $(NH_4)_2SO_4$ saturation by addition of saturated $(NH_4)_2SO_4$ solution adjusted to pH 7.0. The suspension was centrifuged. The supernatant was brought to 57% $(NH_4)_2SO_4$ saturation. The suspension was centrifuged. The pellet was dissolved in 0.1 M phosphate buffer, pH 7.0 and dialyzed overnight against 0.05 M Tris-HCl buffer, pH 8.0. The dialyzed solution was applied to a column of DEAE-cellulose (Whatman DE-52, 2.5 × 27 cm, equilibrated with Tris-HCl buffer, pH 8.0). The column was washed with the same buffer and subsequently developed with a linear gradient (0–0.15 M) of NaCl in 0.05 M Tris-HCl buffer, pH 8.0. Fractions of 10 ml were collected. Deaminase was eluted at a molarity of about 0.075 M NaCl. Fractions were pooled and concentrated by ultrafiltration (Amicon PM-10 membrane).

Results

Deaminase was partially purified by the procedure described under Materials and Methods. A typical experiment is summarized in Table I. The pH optimum of the partially purified enzyme was at 7.3. Addition of EDTA inhibited the activity of the deaminase completely. Enzyme treated with EDTA was restored to 50% of the original activity by the addition of 5 mM Mg^{2+}. The addition of mercaptoethanol during purification led to a reduction of enzyme yields; thus it appears that sulfhydryl reagents reduce the stability of the enzyme. On the other hand, the presence of dithioerythritol is absolutely required in the assay mixture, probably to protect the substrate rather than the enzyme.

In order to identify the product of the enzyme, deaminase (126 U) was incubated for 2.5 h at 37°C with substrate (2.4 μmol) in 0.5 ml of a solution containing 0.1 M phosphate buffer, pH 7.0/10 mM $BeCl_2$/5 mM $MgCl_2$/10 mM dithioerythritol. Diacetyl was added to a final concentration of 0.5 M and the solution was incubated for 15 min at 37°C. The reaction mixture was centrifuged and the supernatant was subjected to paper chromatography. The chromatogram showed a green fluorescent band (R_F = 0.23), subsequently identified as 6,7-dimethyl-8-ribityllumazine 5′-phosphate (VII), and a blue-green fluorescent band (R_F = 0.30), subsequently identified as 2-amino-6,7-dimethyl-4-oxo-8-ribitylpteridine 5′-phosphate (VIII). Bands were localized in ultraviolet light, cut and eluted with water. The yields of 6,7-dimethyl-8-ribityllumazine 5′-phosphate (VII) and 2-amino-6,7-dimethyl-4-oxo-8-ribitylpteridine 5′-phosphate (VIII) were 122 nmol (5.1%) and 125 nmol (5.2%), respectively.

In a control experiment without deaminase, 370 nmol (15%) of 2-amino-6,7-dimethyl-4-oxo-8-ribitylpteridine 5′-phosphate (VIII) were obtained from 2.4 μmol 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5′-phosphate. No 6,7-dimethyl-8-ribityllumazine 5′-phosphate was formed in the control experiment. The 6,7-dimethyl-8-ribityllumazine 5′-phosphate obtained from the enzyme reaction described above was further purified by chromatography on a column of AG 1 × 8 (0.8 × 14 cm, formate form). The column was developed with a linear gradient of formic acid (0.01–2.0 M, 2 × 250 ml) [16]. Fractions of 10 ml were collected. Green fluorescent fractions (21–26) were combined and lyophilized.

TABLE I

PARTIAL PURIFICATION OF 2,5-DIAMINO-6-RIBITYLAMINO-4(3H)-PYRIMIDINONE 5′-PHOSPHATE DEAMINASE FROM *C. GUILLIERMONDII*

Fraction	Protein (mg)	Activity (nmol · h^{-1})	Specific activity (nmol · h^{-1} · mg^{-1})	Purification
Cell extract	2140	89300	41.8	1.0
$(NH_4)_2SO_4$ fractionation	477	48000	101	2.4
DEAE-cellulose	54	31200	578	13.8

TABLE II

ULTRAVIOLET ABSORPTION MAXIMA OF 6,7-DIMETHYL-8-RIBITYLLUMAZINE 5'-PHOSPHATE IN AQUEOUS SOLUTION

Origin	pH	λ_{max}			$(\epsilon_{410}/\epsilon_{259})$
From deaminase reaction	2.0	410	270 (s) [a]	261	0.51
	7.2	410	270 (s)	269	0.51
	12	314	275		
Synthetic	2.0	410	270 (s)	259	0.43
	7,2	410	270 (s)	259	0.43
	12	313	275		

[a] s = shoulder.

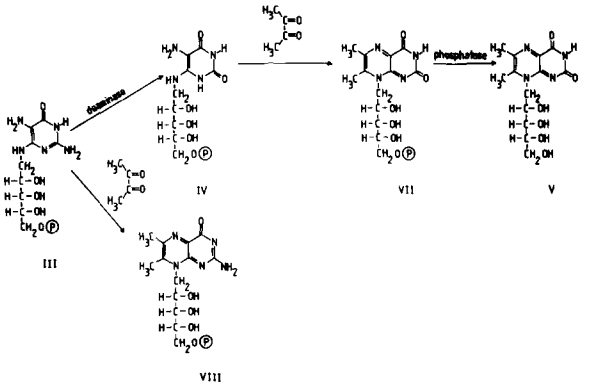

Fig. 2. Chemical and enzymatic conversions of 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5'-phosphate applied in this study. III, 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5'-phosphate; IV, 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5'-phosphate; V, 6,7-dimethyl-8-ribityllumazine; VII, 6,7-dimethyl-8-ribityllumazine 5'-phosphate; VIII, 2-amino-6,7-dimethyl-4-oxo-8-ribitylpteridine 5'-phosphate.

The compound was compared with a synthetic sample of 6,7-dimethyl-8-ribityllumazine-5'-phosphate (VII). The compounds under comparison showed identical absorption spectra (visible and ultraviolet, Table II). They migrated to the same distance in thin layer chromatography under a variety of experimental conditions (Table III, solvent systems I–IX). They also migrated at the same velocity in cellulose acetate electrophoresis at pH 6.8.

A sample of 6,7-dimethyl-8-ribityllumazine 5'-phosphate (VII) produced by enzymatic deamination of 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5'-phosphate (III) and subsequent reaction with diacetyl was treated with alkaline phosphatase (Fig. 2). The compound was converted to 6,7-dimethyl-8-ribityllumazine (V), which was identified by thin layer chromatography (comparison with synthetic sample, solvent systems I–IX). The phosphate content of the 6,7-dimethyl-8-ribityllumazine 5'-phosphate obtained from the deaminase reaction was estimated after hydrolysis catalyzed by alkaline phosphatase [17]. We found 0.94 mol phosphate/mol 6,7-dimethyl-8-ribityllumazine 5'-phosphate (VII). It has been shown previously that 6,7-dimethyl-8-ribityllumazine 5'-phosphate (VII) binds to apoflavodoxin from *Peptostreptococcus elsdenii* [16]. A sample of 6,7-dimethyl-8-ribityllumazine 5'-phosphate (VII) produced by the deaminase action was titrated with apoflavodoxin (courtesy of Prof. S. Ghisla, Constance).

On adding to the compound a 3-fold excess of

TABLE III

R_F VALUES OF COMPOUNDS STUDIED

Thin layer chromatography was performed with precoated cellulose or silica gel plates using the solvent systems indicated. I, 3% NH_4Cl/cellulose; II, *n*-butanol/acetic acid/water (50 : 15 : 35, v/v)/Cellulose; III, *n*-butanol/ethanol/water (50 : 20 : 30, v/v)/Cellulose; IV, collidine/water (75 : 25, v/v)/Cellulose; V, tert. butanol/water (60 : 40, v/v)/Cellulose; VI, *n*-butanol/acetic acid/water (50 : 15 : 35, v/v)/Kieselgel 60; VII, *n*-butanol/ethanol/water (50 : 20 : 30, v/v)/Kieselgel 60; VIII, collidine/water (75 : 25, v/v)/Kieselgel 60; IX, tert. butanol/water (60 : 40, v/v)/Kieselgel 60.

	I	II	III	IV	V	VI	VII	VIII	IX
6,7-Dimethyl-8-ribityllumazine 5'-phosphate (VII)	0.80	0.27	0.23	0.0	0.60	0.13	0.31	0.33	0.63
2-Amino-6,7-dimethyl-4-oxo-8-ribitylpteridine 5'-phosphate (VIII)	0.80	0.30	0.25	0.0	0.38	0.11	0.22	0.19	0.43
6,7-Dimethyl-8-ribityllumazine	0.70	0.42	0.28	0.37	0.40	0.30	0.40	0.83	0.65

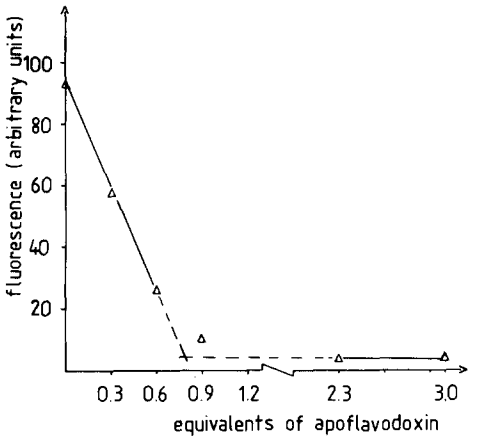

Fig. 3. Fluorimetric titration of 6,7-dimethyl-8-ribityllumazine 5′-phosphate (VII) with apoflavodoxin from *P. elsdenii* (courtesy of Professor S. Ghisla, Constance). 6,7-Dimethyl-8-ribityllumazine 5′-phosphate was obtained by enzymatic deamination of 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5′-phosphate (III) followed by chemical condensation with diacetyl. Excitation, λ = 410 nm, emission, λ = 490 nm.

apoflavodoxin, the fluorescence emission of the lumazine chromophore was quenched to about 4% (Fig. 3). It has been reported that 6,7-dimethyl-8-ribityllumazine 5′-phosphate (VII) is no substrate for riboflavin synthase [16]. In agreement with our earlier report, we found that the isolated 6,7-dimethyl-8-ribityllumazine 5′-phosphate is not metabolized by heavy riboflavin synthase from *Bacillus subtilis*. However, riboflavin was formed by the subsequent action of phosphatase and riboflavin synthase (80% of the theoretical yield).

Discussion

It is known that the early steps of riboflavin biosynthesis involve phosphorylated intermediates [6,7, 9]. On the other hand, it has been shown that the terminal step catalyzed by the enzyme, riboflavin synthase, involves the unphosphorylated substrate, 6,7-dimethyl-8-ribityllumazine (V) [16]. Loss of the phosphoric acid residue could occur in the reaction catalyzed by the pyrimidine deaminase or in a subsequent step. This question could not be resolved by our earlier studies on 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5′-phosphate deaminase due to the presence of a high phosphatase activity in cell extracts of *C. guilliermondii*. In the partially purified enzyme preparation described in this study, the activity of alkaline phosphatase was considerably reduced as compared to the cell extract (18% residual phosphatase activity). Experiments with this preparation were performed in the presence of $BeCl_2$, a known inhibitor of yeast alkaline phosphatase [18].

5,6-Diamino-pyrimidine type compounds are very labile and cannot be easily isolated in substance. However, they can be transformed to pteridine derivatives by the addition of diacetyl. This technique which had proved successful in earlier studies [1–3] was used for the characterization of the product of the deaminase reaction. Incubation of the partially purified deaminase with 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5′-phosphate (III) followed by the addition of diacetyl allowed the isolation of a green fluorescent compound which was identified as 6,7-dimethyl-8-ribityllumazine 5′-phosphate (VII) by comparison with a synthetic sample by a variety of experimental techniques. Enzymatic hydrolysis yielded approximately equimolar amounts of inorganic phosphate and 6,7-dimethyl-8-ribityllumazine (V). The isolated 6,7-dimethyl-8-ribityllumazine 5′-phosphate (VII) binds to apoflavodoxin as shown by quenching of the fluorescence of the lumazine chromophore. Apoflavodoxin has been shown to be highly selective for 5′-phosphorylated riboflavin derivatives, whereas 4′-isomers are not bound [19, 20]. We conclude that the enzyme produces 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5′-phosphate (IV) which was non-enzymatically converted to 6,7-dimethyl-8-ribityllumazine 5′-phosphate (VII) by the addition of diacetyl.

Due to the very low stability of 5,6-diaminopyrimidine type compounds, only a relatively small fraction of pyrimidine 5′-phosphates could be recovered as pteridines in the experiments described. However, the ratio of residual substrate and product formed was about 1 : 1 after incubation with enzyme, thus indicating that about half of the proffered substrate was deaminated under the experimental conditions.

Shavlovskij and coworkers [21] have recently reported on the action of crude cell extracts from *C. guilliermondii* on GTP. Subsequent to treatment of the reaction mixture with diacetyl, they observed phosphorylated derivatives of lumazine and pteridine.

Whereas the authors give no detailed structural proof for the isolated compounds, their data are consistent with the biosynthetic scheme in Fig. 1.

Acknowledgements

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