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NAD+-DEPENDENT FORMATION OF 2-AMINO-4-HYDROXY-6-CARBOXYPTERIDINE FROM 2-AMINO-4-HYDROXY-6-FORMYLPTERIDINE BY CELL-FREE EXTRACTS OF *ESCHERICHIA COLI*

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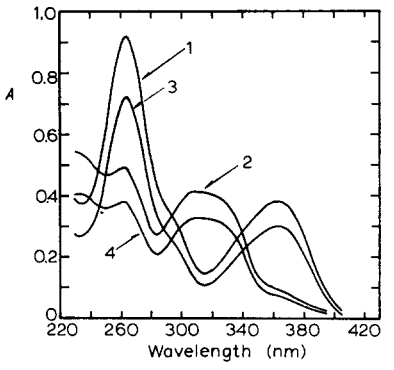
SUMMARY

Cell-free extracts of *Escherichia coli* B catalyze the production of 2-amino-4-hydroxy-6-carboxypteridine from 2-amino-4-hydroxy-6-formylpteridine. NAD^+ is essential as a cofactor for this reaction. The product is isolated by column chromatography on Sephadex G-25 and by paper partition chromatography. The structure of the product is determined on the basis of ultraviolet spectra, behavior on paper chromatography, and pH–fluorescence curves.

2-Amino-4-hydroxy-6-carboxypteridine has been widely isolated from various living organisms[1], and is supposed to arise from 2-amino-4-hydroxy-6-formylpteridine, a primary degradative product from folic acid[2]. This conversion actually happens under catalysis with xanthine oxidase (xanthine:O_2 oxidoreductase, EC 1.2.3.2)[3]. However, the reaction is very slow, and the enzyme is also most strongly inhibited by very low concentrations of the formylpteridine[4–8]. This evidence necessarily makes it difficult to accept that xanthine oxidase plays a main role in the formation of the carboxypteridine *in vivo*. Any effective reaction catalyzed by an enzyme (or enzymes) except xanthine oxidase has not been found yet. The present communication demonstrates that the formylpteridine is efficiently transformed to the carboxypteridine by an enzymatic reaction dependent on NAD^+ in *Escherichia coli* B.

2-Amino-4-hydroxy-6-formylpteridine[9] and 2-amino-4-hydroxy-6-carboxypteridine[10] were prepared by the published methods. The enzyme preparation used was the dialysate of the protein precipitate at 80% $(NH_4)_2SO_4$ saturation from cell-free extracts of *E. coli* as described previously[11].

Isolation and characterization of the enzymic reaction product was attempted. The reaction medium (9 ml) contained 1.8 μmole 2-amino-4-hydroxy-6-formylpteridine, 2.7 μmoles NAD^+, 450 μmoles phosphate buffer (pH 7.0), and 12.2 mg of enzyme protein. After being kept at 37° for 3 h in the dark, the medium was immediately applied on a Sephadex G-25 column (coarse, 2.8 cm × 50 cm) previously washed with distilled water, and developed with water. 10-ml fractions were collected at a rate of 10 ml/20 min. The blue fluorescent fractions (Tubes 22–25) under ultraviolet



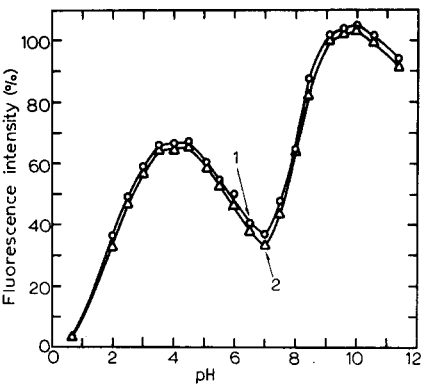


Fig. 1. Ultraviolet absorption spectra of 2-amino-4-hydroxy-6-carboxypteridine (35.6 μM) and the enzymic product from 2-amino-4-hydroxy-6-formylpteridine. The spectra were recorded using a Shimazu recording spectrometer. Curves 1 and 2, spectra of the enzymic product in 0.1 M NaOH and HCl, respectively; Curves 3 and 4, spectra of 2-amino-4-hydroxy-6-carboxypteridine in 0.1 M NaOH and HCl, respectively.

Fig. 2. pH–Fluorescence curves of 2-amino-4-hydroxy-6-carboxypteridine and the enzymic product from 2-amino-4-hydroxy-6-formylpteridine. The concentration of the carboxypteridine (0.425 μM) was chosen so that the maximum fluorescence at pH 9.1 gave a scale reading of 100% on a Shimazu-Kotaki ultramicro fluorophotometer type UM. The buffers used were the same as those used by KAVANAGH AND GOODWIN[12]. Curve 1, the enzymic product; Curve 2, 2-amino-4-hydroxy-6-carboxypteridine.

ight were combined, and evaporated at 45° *in vacuo* to 5 ml. This solution was re-chromatographed once more on the same column. The concentrate of the fluorescent product was streaked on large sheets of filter paper (Tôyô filter paper No. 51, 20 cm × 29 cm, 8 sheets). The chromatograms were developed by the ascending method with a solvent of abs. ethanol–water (1:3, by vol.). The fluorescent band (R_F = 0.83) on each chromatogram was eluted with boiled water. The eluate was evaporated at 45° *in vacuo*, and was chromatographed on the Sephadex column as described before. The fluorescent fractions were combined, and concentrated at 45° *in vacuo* to a small volume, which was used as an analytical sample. All operations described above were performed at room temperature in the dark. The blue fluorescent product arising from 2-amino-4-hydroxy-6-formylpteridine was essentially identical with authentic 2-amino-4-hydroxy-6-carboxypteridine with respect to ultraviolet absorption spectra in 0.1 M HCl and NaOH (Fig. 1), the pH-fluorescence curve obtained at pH levels from 0.7 to 11.5 (Fig. 2), and R_F values on paper chromatograms developed with various solvent systems. Development of the chromatogram (Tôyô filter paper No. 51) at room temperature was carried out by the ascending method. The R_F values of the enzymic product were as follows: R_F = 0.70 in abs. ethanol–water (1:3, by vol.); R_F = 0.37 in 4% sodium citrate; R_F = 0.22 in *n*-butanol–acetic acid–water (4:1:5, by vol., upper phase); R_F = 0.16 in *n*-propanol–1% NH_4OH (2:1, by vol.); and R_F = 0.57 in 3% NH_4Cl.

As shown in Table I, the enzymic conversion of 2-amino-4-hydroxy-6-formylpteridine to 2-amino-4-hydroxy-6-carboxypteridine was absolutely dependent on the presence of NAD^+. The NAD^+ requirement could not be replaced by $NADP^+$.

Two separate reactions for the conversion of 2-amino-4-hydroxy-6-formyl-

TABLE I

REQUIREMENT OF NAD^+ FOR THE ENZYMIC CONVERSION OF 2-AMINO-4-HYDROXY-6-FORMYLPTERIDINE TO 2-AMINO-4-HYDROXY-6-CARBOXYPTERIDINE

Reaction mixture contained, per 0.5 ml, 0.041 μmole 2-amino-4-hydroxy-6-formylpteridine, 0.075 μmole NAD^+, 25 μmoles potassium phosphate (pH 7.0), and 3.1 mg of enzyme protein. Incubation at 37° proceeded for 30 min. Reaction was stopped by adding 0.5 ml of 0.1% 2,4-dinitrophenylhydrazine dissolved in 2 M HCl. The residual formylpteridine was determined by the method of KALCKAR *et al.*[3] Concentration of $NADP^+$ was identical with that of NAD^+.

Reaction system	*Consumption of 2-amino-4-hydroxy-6-formylpteridine (nmoles)*
Complete	27.0
Minus NAD^+	2.0
Minus NAD^+, *plus* $NADP^+$	0
Minus enzyme	1.3

pteridine to 2-amino-4-hydroxy-6-carboxypteridine might be possible: (a) one catalyzed by oxidases such as xanthine oxidase and aldehyde oxidase (aldehyde:O_2 oxidoreductase, EC 1.2.3.1), and (b) the other catalyzed by aldehyde dehydrogenases requiring NAD^+ or $NADP^+$ as a cofactor. As mentioned before in this paper, it seems implausible that pathway (a) operates *in vivo*. BYERS[13], and DIETRICH *et al.*[14] demonstrated that the formylpteridine was rapidly metabolized *in vivo* without any influence on xanthine oxidase activity. However, no conclusive evidence has ever been provided yet for the formation of the carboxypteridine catalyzed by aldehyde dehydrogenases. The present work is the first demonstration of such a type of reaction. As shown in Table I, the enzymic conversion of the formylpteridine to the carboxypteridine is a NAD^+-specific reaction, not dependent on $NADP^+$. The reaction does not occur when NAD^+ is absent from the reaction system. These findings indicate that a NAD^+-specific aldehyde dehydrogenase, and not oxidases, functions mainly for the conversion in *E. coli*.

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