Enzymatic Studies of Riboflavin Oversynthesis in 
_Eremothecium ashbyii_

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Summary  Mechanisms of riboflavin oversynthesis in a high flavinogenic mold, _Eremothecium ashbyii_, were examined in relation to growth, riboflavin formation and related synthases, and medium pH with increasing culture periods. Growth reached maximum at 1 d and then decreased, riboflavin formation proceeded rapidly up to 5 d and approached almost a plateau region. The medium pH reached minimum at 1 d and thereafter fairly rapidly increased until 3 d, then gradually increased to 7 d after cultivation. The crude enzyme solution from the mycelia at specified culture periods was run through a column of Sephadex G-200, indicating two riboflavin synthase activities on the chromatogram. The fluctuation of the growth and the specific activities of the two enzymes were examined with increasing culture periods, which showed that the heavy enzyme may be a constitutive one and that the light enzyme may be concerned with the oversynthesis of riboflavin in _E. ashbyii_. The heavy enzyme was then purified by 49-fold after dialysis of the ammonium sulfate precipitate by a series of column chromatographies with Sephadex G-200, hydroxyapatite, DEAE-Sepharose A-50 and DEAE-cellulose. The purified enzyme which was treated with weak alkaline solution was broken into the light enzyme, showing two bands on an acrylamide disc gel electrophoresis. The relation of the heavy and the light enzymes to the oversynthesis of riboflavin in _E. ashbyii_ was discussed.

Key Words  riboflavin, oversynthesis, _Eremothecium ashbyii_, riboflavin synthase

Overview of the biosynthetic pathway was substantially done in 1980 (1, 2). Since then, the detailed reaction mechanisms between the primary intermediates have been further investigated (3). At the gene level, the operon involved in the riboflavin biosynthesis has been detected in _Helicobacter pylori_ (4), _Photobacterium sp._ (P. leiognath (5), _P. phosphoreum_ (6)), _Bacillus subtilis_ (7) and yeast (7).

Used widely and for a long time in the industrial production of riboflavin as well as in these studies (8), a high flavinogenic ascomycetes, _Eremothecium ashbyii_, was employed because of the formation of prodigious amounts of riboflavin. We had also determined the immediate purine (9) and nucleotide precursor (10), the intermediates (11, 12), the by-product in the riboflavin synthase reaction (13), and postulated the recycling of the by-product in the riboflavin synthetase reaction (14), using the resting cells of _E. ashbyii_. However, the mechanisms of the oversynthesis in the high flavinogenic mold, _E. ashbyii_, have never been elucidated. We tried here to clarify, enzymatically, the mechanisms of the overproduction in the high flavinogenic mold.

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MATERIALS AND METHODS

**Materials.** The reagents for fermentation of the microorganisms and the chemicals for electrophoresis, acrylamide, N,N'-methylene-diamine-acrylamide (Bis), N,N,N',N'-tetramethylethylene-diamide (TEMED), Amido Schwarz, etc., were purchased from Wako Pure Chemical Industries, Co., Ltd., Japan. The resins for preparation and purification of the substrate, 6,7-dimethyl-8-ribityllumazine (DMRL), and of the enzymes, Dowex 1×2, -50 W×4 (200 to 400 mesh) (Dow Chemical Co., Midland, MI, USA), Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden), Hydroxyapatite (Wako Pure Chemical Industries), DEAE-Sepharose-A-50 (Pharmacia Fine Chemicals), and DEAE-Cellulose (Serva AG, Heidelberg, Germany) were employed. The other chemicals used were of the best quality available.

**Organisms.** The mold, _Eremothecium ashbyii_ IFO 0944, was purchased from The Institute of Fermentation Osaka Co., Ltd., Japan. A riboflavin-adenine-deficient mutant, _Bacillus subtilis_ AJ1988, was the gift of Ajinomoto Co., Ltd., Japan.

**Medium and fermentation.** The fermentation media and fermentation of _E. ashbyii_ were the same as in previous paper (15). A loopful of the mycelia on an agar slant was subsequently transplanted onto the precul-
Preparation of crude DMRL, for determination of the synthase activity in the eluates from the columns. The preparation of the crude substrate, DMRL, was done as follows: The supernatant (300 mL) was applied to a double-layered column (3.6 × 80 cm) of Dowex 50 W×4 (H⁺) and Dowex 1×2 (HCOO⁻) and eluted with distilled water. The green fluorescent fractions were collected under ultraviolet light. The concentration of the crude DMRL solution was calculated from the absorbances at 280 nm.

Preparation of crude enzyme from the mycelia of E. ashbyii. The extraction of the enzyme was done as previously reported (15). The mycelia of E. ashbyii obtained at specified periods were washed with alumina. To the slurry, the phosphate buffer (pH 6.8) was added and centrifuged. The ammonium sulfate was supplemented to 80% saturation to the supernatant. The precipitate obtained was centrifuged to obtain the supernatant containing a large amount of DMRL.

Preparation of crude enzyme from the mycelia of E. ashbyii. The extraction of the enzyme was done as previously reported (15). The mycelia of E. ashbyii obtained at specified periods were ground with alumina. The green fluorescent fractions were collected under ultraviolet light. The concentration of the crude DMRL solution was calculated from the absorbances at 280 nm for DMRL. An appropriate volume of the solution was used for detecting riboflavin synthase activity in the eluates from the various columns.

Preparation of crude enzyme from the mycelia of E. ashbyii. The extraction of the enzyme was done as previously reported (15). The mycelia of E. ashbyii obtained at specified periods were ground with alumina. To the slurry, the phosphate buffer (pH 6.8) was added and centrifuged. The ammonium sulfate was supplemented to 80% saturation to the supernatant. The precipitate obtained was centrifuged to obtain the supernatant containing a large amount of DMRL.

The protein amounts in the eluate from Sephadex G-200 column (Fig. 2) were expressed in terms of the absorbances at 280 nm.

Enzyme assay. The enzyme activities in each fraction from the Sephadex G-200 column (Fig. 2) and from a series of column used for purification of the heavy riboflavin synthase were determined in the mixture containing 0.6 mL of 0.1 M potassium phosphate buffer (pH 6.8), 0.6 mL of 0.103 mM crude DMRL solution and 0.6 mL solution in each fraction. The reaction was performed in darkness at 27°C for 30 min and terminated by the addition of 0.5 mL of 30% trichloroacetic acid. After the centrifugation of the reaction mixture, the absorbance at 408 and 470 nm of the supernatant were estimated and extrapolated into the following equation for calculating the amounts (nmol/mL) of formed riboflavin: Absorbance at 470 nm × 104.6 - Absorbance at 405 nm × 0.670 × 1.5 (17). The obtained riboflavin was employed as a candidate for assessing the synthase activity in each fraction from the Sephadex G-200 column in Fig. 2 and from a series of column for purification of the heavy synthase. The specific activities of the enzymes on the Sephadex G-200 column chromatograms at specified days (Fig. 2) and on various column chromatograms were calculated using the riboflavin amounts obtained by the same enzyme reaction as above with the purified DMRL in place of the crude DMRL and using the protein amounts measured by the method of Lowry et al. (18), and expressed in terms of nano mole riboflavin/mg protein (1 unit).

The heavy riboflavin synthase was purified 49-fold in terms of the specific activity by the purification steps from the dialysis of an 80% ammonium sulfate precipitate.

Acrylamide disc gel electrophoresis. The purified heavy enzyme solution obtained as above was concentrated, adjusted to pH 8.0 and left at 5°C for several hours. An appropriate volume of the enzyme solution was subjected to the same acrylamide disc gel electrophoresis (pH 8.9) (19) except for the chemical, in part, and the electrical conditions, using an instrument (SJ-1060DC II, ATTO, Co., Ltd., Osaka). The gel for concentration consisted of acrylamide, Tris-HCl buffer (pH 6.8), ammonium persulfate, distilled water and TEMED (2.993 mL in total, acrylamide 4.0% in final). The gel for separation contained acrylamide, Tris-HCl buffer (pH 8.8), ammonium persulfate, distilled water and TEMED (9.661 mL in total, acrylamide 7.5% in final). After application of the enzyme solution treated as above to the upper layer of the acrylamide disc gel, the electrophoresis was performed under the electrical conditions of 2 mA, 100 V and 5 mA, 100 V for about 30 min. The protein bands on the gel were dyed in Amido Schwarz-acetic acid solution. The blue-colored disc gel was immersed in an acetic acid solution and decolorized.

The synthase activities of two bands were confirmed by a simultaneous disc gel electrophoresis.
Mechanisms of Riboflavin Oversynthesis in *E. ashbyii*

**Fig. 1.** Fluctuation of growth, riboflavin formation and medium pH with increasing culture periods. These parameters were determined in a basal medium with increasing rearing periods of *E. ashbyii*. For experimental conditions see details in the Materials and Methods section.

**RESULTS**

*Growth and riboflavin formation in the culture of Ere- mothecium ashbyii.* The growth, riboflavin formation and medium pH in *E. ashbyii* were examined relative to culture periods. Results are given in Fig. 1. The growth reached maximum on the 1st day and thereafter continued to decrease until the end of the experiments (7 d). In contrast, the riboflavin formation vigorously increased on the 1st day, rapidly up to the 5th day in a straight line and reached almost a plateau region at the 5th day. The pH values slightly decreased on the 1st day, then increased up to the 3rd day and thereafter gradually increased to the end of the experiments.

*Relation of heavy and light riboflavin synthase to ribofla- vin formation with the culture of E. ashbyii.* An appropriate volume of the dialyzed enzyme solution at specified culture periods was applied onto a column of Sephadex G-200 and eluted. The results are shown in Fig. 2. On the 1st day, the proteins were sharply eluted in the fractions from 9 to 13 and thereafter gradually eluted to the end of the experiments. The riboflavin synthase activities were found in the fractions from 11 to 12 and 19 to 24 in significantly large amounts. On the 2nd day, the elution pattern of proteins was almost the same as that at the 1st day. The riboflavin synthase activities were found in the fractions of 9 and from 17 to 22 in large amounts. On the 3rd day, the proteins were eluted in the same way as on the 1st and 2nd days. The synthases were found in the fractions from 8 to 10 in trace amounts, and from 17 to 22 in large amounts. On the 5th day, the elution pattern of proteins was also obtained as in previous days, and the synthase activities were detected in the fraction of 11 and from 19 to 23 fractions in small amounts. On the 7th day, the elution pattern of proteins was found similarly, but at the lower levels, and both synthase activities were found in trace amounts on the chromatogram.

**Fig. 2.** Fluctuation of the riboflavin synthase activity and protein amounts on the chromatograms of Sepha- dex G-200 with increasing culture periods. The mycelia collected at specified times were ground and centrifuged. The supernatant was applied to a column of Sephadex G-200 and eluted with 0.1M potassium phosphate buffer (pH 6.8). For experimental conditions see details in the Materials and Methods section.

**Fig. 3.** Fluctuation of specific activities of both ribofla- vin synthases with increasing culture periods. For ex- perimental conditions see details in the Materials and Methods section.
Fig. 4. Electrophoretic pattern of riboflavin synthases on an acrylamide disc gel. An appropriate volume of purified enzyme solution treated with alkaline solution was loaded on the upper layer of disc gel concentration column and subjected to electrophoresis, and decoloration was then performed. For experimental conditions see details in the Materials and Methods section.

**DISCUSSION**

Recently, regulation of the early pathway of riboflavin formation has been reported accomplished by feedback inhibition of the end product in *B. subtilis* (20), *Pichia guilliermondii* (21), and by repression due to iron in *P. guilliermondii* (20). In contrast, the mechanisms of riboflavin oversynthesis in the riboflavin-overproducing microorganisms have been investigated in relation to the change in oxidative pathways from the cytochrome type to the flavoprotein type (22), and to oxidative stress with or without iron in *P. guilliermondii* (23) and to mutation of the regulatory gene, rib 83 (24) and ribC (20) of riboflavin formation in *P. guilliermondii* and *B. subtilis*, respectively. Because of its significant oversynthesis of riboflavin (8), the mold *E. ashbyii* is frequently used in industrial production (7) and in studies elucidating the biosynthetic pathway of riboflavin (1). This may indicate that riboflavin does not inhibit any biosynthetic pathway of riboflavin in *E. ashbyii*, although riboflavin is reported to inhibit the activity of DMRL synthase in *P. guilliermondii*, although riboflavin is reported to inhibit the activity of DMRL synthase in *P. guilliermondii* (25), to significantly reduce the DMRL oversynthesis by the supplementation of high concentrations (0.5 μg/mL) of riboflavin to the riboflavin-deficient mutant of *B. subtilis* (Nakajima and Yakushigawa, unpublished data), and to regulate the transcription level of riboflavin biosynthesis in *B. subtilis* SH gw (26). The fact that riboflavin has no effect on riboflavin formation was also confirmed by the supplementation experiments with large amounts (150 μg/mL) of riboflavin to the resting cells of *E. ashbyii* (Nakajima, unpublished data) being permeable to exogenous riboflavin (27). Therefore, the regulation mechanism of the riboflavin oversynthesis in *E. ashbyii* remains obscure.

The two enzymes, heavy and light enzymes, catalyzing the riboflavin formation have been detected in several bacteria (28). The two enzymes in the ascomycetes, *E. ashbyii*, were first found in the present experiments, but the occurrence of two enzymes may need to be carefully examined because of the easy degradation of the heavy enzyme to the light enzyme. The purified heavy enzyme was easily degraded to the light enzyme in the weak alkaline solution at cold temperature, also in the present paper. In *B. subtilis*, the heavy enzyme breaks into the light enzyme under milder conditions that have a higher characteristic property (about 1/10 fold Km values against those of the heavy enzyme) (28). In the present experiments, the specific activity of the heavy enzyme and the growth showed their maximum on the 1st day. In contrast, the specific activity of the light enzyme reached maximum on the 3rd day, showing a parallel relation to the riboflavin formation to the culture time. These may suggest that the heavy enzyme is a constitutive enzyme needed for attaining the maximal growth at the 1st day and the light enzyme is concerned with the oversynthesis of riboflavin to the 3rd day in *E. ashbyii*. During the culture period for 3 d, the pH values of the medium rapidly increased from 2 to 3 d (alkaline medium) and thereaf-
The point mutation of the regulatory gene, ribC, causes the oversynthesis of riboflavin in *B. subtilis* (31). The encoding protein of ribC is reported to show a significant sequence similarity to riboflavin kinase and FAD-synthase in *B. subtilis* (30). In the process of over-synthesis of riboflavin in *E. ashbyii*, FMN and FAD are normally formed in amounts comparative to riboflavin in the mycelia (32, 33). Accordingly, riboflavin kinase and FAD synthase possibly being regulatory proteins appear not to operate as the regulator of riboflavin formation in *E. ashbyii*. The products, FMN and FAD, also appear not to restrict the activities of GTP cyclohydro-lase involved in the first step of riboflavin formation.

Accordingly, an ingenious enzymatic mechanism of flavin biosynthesis is believed to operate in a high flavinogenic mold, *E. ashbyii*.

The purification and properties of the heavy riboflavin synthase and the nature of the regulatory gene on the riboflavin-operon in *E. ashbyii* need to be enzymatically and genetically elucidated in the future.

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