CATALYTIC PROPERTIES OF RIBOFLAVIN SYNTHETASE FROM A HIGH-RIBOFLAVINOGENIC *EREMOTHECIUM ASHBYII*¹

Yuzuru SUZUKI,² Yoshiyuki NISHIKAWA, and Hisateru MITSUDA³

*Laboratory of Nutritional Chemistry, Faculty of Agriculture,
Kyoto University, Sakyo-ku, Kyoto 606*

(Received June 6, 1974)

Riboflavin synthetase catalyzes a conversion of two molecules of 6,7-dimethyl-8-ribityllumazine to one molecule each of riboflavin and 4-ribitylamino-5-aminouracil. A riboflavinogenic *Eremothecium ashbyii* produces this enzyme only within an earlier stage of the growth phase of its fungal life cycle prior to an initiation of vitamin overproduction, and the enzyme persists to function throughout the cycle. The specific enzyme activity of the cell-free extract is high, 72 to 165 μ moles of riboflavin formed per hour per mg of protein at 37°C, approximately 100 times higher than those of the vitamin-producing microbes examined to date.

The enzyme exhibited a broad plateau on its activity curve between pH of 5.7 and 7.0 with a maximum point at pH 6.5. The optimal activation with temperature occurred at 47°C, but the enzyme was completely inactivated at 60°C. The apparent activation energy was 11,000 calories per mole between 42° and 20°C. The initial reaction velocity showed a second order dependency on the lumazine concentration in the range 1.5×10^{-4} to 6.3×10^{-6} M, *i.e.*, a sigmoidal kinetic response instead of a hyperbolic Michaelis-Menten behavior, suggesting that affinities of the two substrate binding sites present on the enzyme have similar orders of magnitude. A Hill plot of the velocity consists of two straight lines with a slope=2 at lumazine levels below 6.0×10^{-5} M and with a slope=1 at the levels beyond this value. When the lumazines were bound to the enzyme in a random order, $Ka' + Kd'$ and $Ka'Kd$ (or $KaKd'$), where Ka' and Kd' denote the dissociation constants of a ternary enzyme-substrate complex and Ka and Kd are the constants of

¹ An outline of this paper was read at the annual meetings of the Kinki Branch of the Japanese Biochemical Society (May 29, 1971) and the Vitamin Society of Japan (May 21, 1971).

² Present address: Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto Prefectural University, Shimogamo, Sakyo-ku, Kyoto 606.

³ 鈴木 譲, 西川善之, 満田久輝

the two binary complexes present, were calculated as 4.5×10^{-5} M and 2.7×10^{-9} M², respectively, while, when an ordered mechanism held, Kd and Ka' (or Ka and Kd') corresponded to the respective values of 6.0×10^{-5} and 4.5×10^{-5} M. A reaction mechanism without a kinetically significant ternary complex was not the case for *E. ashbyii* enzyme.

A simplified method for purification of riboflavin synthetase from *E. ashbyii* is reported. The specific enzyme activity of the purified preparation was 6,370 mμmoles of the vitamin produced for one hour per mg of protein at 37°C.

Riboflavin synthetase catalyzes the direct formation of one molecule of riboflavin from two molecules of 6,7-dimethyl-8-ribityllumazine. HARVEY and PLAUT (1) have proposed the presence of two substrate-binding sites on the enzyme. The donor site binds the lumazine in such a way that it functions as a donor for the 4-carbon moiety to be transferred in the reaction, and the acceptor site binds the lumazine that serves as the acceptor of the 4-carbon fragment. The rate equation formulated from such a mechanism contains a term, second-order, with respect to the substrate concentration, indicating a possible nonlinear relationship between the reciprocals of velocity and the substrate concentration. Such a relationship, however, has never been reported until a preliminary investigation from this laboratory was presented recently (2).

The reaction catalyzed by riboflavin synthetase from yeast (1), spinach (3) and *Ashbya gossypii* (4) has been known to proceed under zero- to first-order kinetics. But, the catalytic reaction by the enzyme from a high-riboflavinogenic *Eremothecium ashbyii* shows a second-order dependency of the reaction velocity on the lumazine concentration (2). On the basis of this finding, the present authors suggested (2) that *E. ashbyii* enzyme differs markedly from the other enzymes with respect to its affinity for the substrate and the fundamental protein conformation at or around the donor site. The present report demonstrates that the level of riboflavin synthetase in the mycelia of the mold is extraordinarily high compared with those observed in the other organisms mentioned above. Also, some catalytic properties and an isolation procedure for the enzyme are described in this report.

EXPERIMENTAL

Materials. The 6,7-dimethyl-8-ribityllumazine used was a pure crystalline preparation isolated from mycelia of *E. ashbyii*, which was the kind gift of the late Dr. S. Kuwada. Peptone was purchased from Kyokuto Pharmaceutical Industries Ltd., yeast extract from Oriental Yeast Industries Ltd., and highly purified agar from Difco Laboratories.

Organisms. *E. ashbyii* strain IFO 0944 was obtained through the courtesy of Institute for Fermentation, Osaka. The mold was periodically transferred from a 7-day culture to fresh agar slants (pH 6.5) containing 1% glucose, 1% peptone, 0.1% yeast extract, 1.2% agar, and distilled water. All cultivations were made at 27°C, and sterilization of all media was conducted by autoclaving at 15 pounds of pressure for 15 min.

Growth and determination of mycelial weight and riboflavin. The basal medium (pH 5.5) contained 2% glucose, 1.8% Tween 80, 1% peptone, 0.3% yeast extract, 0.2% KH_2PO_4 , 0.1% NaCl , 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and distilled water. Nutrients in inoculum were the same as the slants, except for agar. Mycelial fragments from the 7-day old slants were suspended in 50 ml of the inoculum in a 300-ml Erlenmeyer flask and allowed to grow under agitation on a rotatory shaker for 24 hr at 220 cycles per minute. Ten ml of the seed culture were inoculated into 500 ml of the basal medium in a 2-liter Erlenmeyer flask. Growth was allowed to proceed in the dark for the defined period with continuous shaking at 220 cycles per minute. At the end of the period, mycelia were separated from the liquid by filtration through a filter paper. After sufficient washing by 2 to 4 liters of cold distilled water at 0 to 4°C, the wet weight of the mycelial pade was determined. The filtrate of the medium was submitted to an estimate of the content of the vitamin formed, evaluated photometrically by reading absorbancy at 450 nm as described in a previous report (5).

Enzyme assay. The reaction mixture (1.5 ml) contained 15 μmoles mercaptoethanol, 15 μmoles Na_2SO_3 , 75 μmoles potassium phosphate (pH 7.0), 225 $\text{m}\mu\text{moles}$ 6,7-dimethyl-8-ribityllumazine and enzyme equivalent to about 1 mg of protein. The reaction proceeded for 10 to 30 min at 37°C, and was stopped at the end of the reaction period by adding 0.5 ml of 30% trichloroacetic acid and 1.0 ml of distilled water, followed by centrifugation at 8,000 rpm for 10 min. Riboflavin involved in the supernatant solution was determined by the photometric method of PLAUT (4) using the following equation:

$$\mu\text{moles of riboflavin per ml} = \frac{(A_{470} \times 10,300) - (A_{405} \times 144)}{97,900}$$

where A_{470} and A_{405} are absorbancies at 470 and 405 nm, respectively. Parallel experiments on controls lacking only enzyme or the substrate were run. One unit of enzyme activity was defined as 1 $\text{m}\mu\text{mole}$ of the vitamin formed per hour. Specific activity was expressed in units per mg of protein. Protein concentration was estimated by the procedure of LOWRY *et al.* (6) using serum albumin as a standard, or by the photometric method of KALCKAR (7) by reading the absorbancies at 260 and 280 nm. When the light absorption of riboflavin present in the sample seriously interfered at these wave lengths, a modification was made in the latter method by using the following equation:

$\text{mg of protein per ml} = 1.45 \times (A_{260} - 1.15 \times A_{450}) - 0.74 \times (A_{280} - 2.27 \times A_{450})$,
where A_{260} , A_{280} and A_{450} are respective absorbancies at 260, 280 and 450 nm.

Initial concentration of 6,7-dimethyl-8-ribityllumazine was determined by reading absorbancy at 405 nm, using a molar extinction of 10,300 in the neutral solution (4).

Continuous measurement of riboflavin synthesis. This assay was undertaken following enzyme purification. The reaction medium (1 ml) consisted of 10 μ moles mercaptoethanol, 10 μ moles Na_2SO_3 , 100 μ moles potassium phosphate at pH 6.5, 2 μ moles ethylenediaminetetraacetate sodium salts at pH 7.0, 200 μ moles 6,7-dimethyl-8-ribityllumazine, and enzyme. The control system contained all components except the substrate. Increase of absorbancy at 470 nm accompanying the formation of riboflavin from the lumazine was monitored continuously in 1.0-cm light-path cells in a Shimazu recording spectrophotometer, Model MPS-5000. The cells were maintained at 37°C by circulating temperature-controlled water in the cell holder. The reaction was started by addition of 0.05 ml of the substrate solution into 0.95 ml of the complete reaction medium with the substrate omitted, and the absorption change at 470 nm was recorded against the control for 1 to 2 min. The initial reaction rate was determined from the slope of the record progressing linearly. A molar extinction of 9,468 was utilized in calculating the yield of the vitamin produced.

Extraction and concentration of enzyme. Mycelia of *E. ashbyii* (150 g) harvested from 4.5 liter cultures after growth for 2 days were mixed with alumina (225 g) of bacterial grade (GB-800) and ground for 10 min with a chilled mortar and a pestle. The cell pastes thus obtained were suspended in 0.1 M potassium phosphate buffer at pH 7.0 (300 ml) containing 10 mM Na_2SO_3 and 10 mM mercaptoethanol, and ground further for 10 min. The suspension was centrifuged at 10,000 rpm and 4°C for 30 min. To the extract (330 ml), solid ammonium sulfate was slowly added to give a final saturation of 20%. The pH of the extract was maintained to 6.5 by addition of 1 N NaOH during this operation.

Table 1. Fractionation of riboflavin synthetase activity by ammonium sulfate.

The enzyme activity was determined as described in the text. Each enzyme preparation indicated above was diluted 10- to 40-fold with 0.1 M potassium phosphate buffer at pH 7.0 containing 10 mM Na_2SO_3 and 10 mM mercaptoethanol. A 0.5 ml- aliquote of the diluted solution was submitted for the enzyme reaction and allowed to react for 20 min at 37°C.

Fractions	Total volume (ml)	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg protein)	Recovery of activity (%)
Extract	330	7,160	766,000	107	100
Fractionation with $(\text{NH}_4)_2\text{SO}_4$					
0-20%	18.5	485	12,300	25.3	1.6
20-35%	20.0	2,000	28,700	14.4	3.8
35-50%	24.5	1,270	286,000	225	37.3
50-65%	14.7	875	201,000	230	26.2
65-100%	355	10.7	111	10.4	0.0

After stirring the solution for one hour at 4°C, the precipitate was collected by centrifugation, and dissolved in the same buffer as used in the extraction of the enzyme (Fraction 0–20% saturation). The supernatant fluid (345 ml) was similarly fractionated with solid ammonium sulfate as indicated in Table 1 (Fractions 0–20%, 20–35%, 35–50%, 50–65%, and 65–100% saturation). Both fractions containing proteins precipitated by ammonium sulfate saturation from 35 to 50% and from 50 to 65% were combined. About 64% of the original enzyme activity found in the extract was recovered in the combined solution. Specific activity of this preparation was detected to be 227 units per mg of protein, approximately purified twofold from the extract. This enzyme preparation was utilized throughout the experiments to determine the characteristics of riboflavin synthetase. At this stage of the enzyme purification, the enzyme was stable at least for one month, if it was kept frozen at –20°C.

RESULTS

1. Change of riboflavin synthetase activity during growth of *Eremothecium ashbyii*

Typical features of growth and riboflavin production of *E. ashbyii* under the standard conditions employed are shown in Fig. 1 (Left). The mold grew rapidly

Fig. 1. Change of riboflavin synthetase activity during growth of *Eremothecium ashbyii*. The mold was allowed to grow in five 2-liter Erlenmeyer flasks each containing 500 ml of the basal medium under the conditions described in the text. Each day, throughout the cultivation sequence, a single flask was removed for testing. Mycelial weight, riboflavin yield, riboflavin synthetase activity and protein extracted from the cells were determined as indicated in the text. The cells harvested were ruptured at 0 to 4°C by hand grinding with alumina of bacterial grade (GB-800) (cell: alumina=1:1.5 (weight per weight)). After the mixture was suspended in 0.05 M potassium phosphate buffer at pH 7.0 (cell: buffer=1:2 (weight per volume)), grinding was continued for 15 to 20 min. The suspension was centrifuged at 10,000 rpm and 4°C for 30 min. A 0.125 ml-aliquot of the supernatant liquid was provided as the enzyme preparation for each reaction, which proceeded for 30 min at 37°C.

during the first 24 hours of fermentation, and maximum growth was achieved at 2 days' culture. After this time, the growth stopped, followed by the beginning of mycelial autolysis. On the contrary, vitamin formation started in coincidence with the growth cessation, and was highly stimulated with progressing autolysis. Such unparalleled growth and vitamin production is a striking attribute of the high-flavinogenic strains of *E. ashbyii* (8, 9). However, one can see in Fig. 1 (Right) that riboflavin synthetase catalyzing the final step in the vitamin biosynthesis was found at its highest level when the growth proceeded vigorously. The specific activity of the enzyme detectable in the cell extracts was maximum after 1 day of cultivation, for which the activity was revealed 96 units per mg protein. But, after this time, the activity reduced promptly to about half of the peak value at the end of the fermentation period. The enzyme was obtained with a maximum yield at 2 days' culture, at the peak of the growth, and the yield markedly fell off parallel with the fungal autolysis after 3 days. The final value at 5 days' culture was reduced to 34.6% of the maximum value. These findings indicate definitely that the enzyme is synthesized during a confined period of the fungal life cycle, corresponding to the earlier stage of the rapid growth prior to the vitamin production.

2. Some catalytic properties of riboflavin synthetase

Time course of reaction. The enzymatic formation of riboflavin progressed in a linear fashion for at least 15 min, as shown in Fig. 2. However, the reaction rate after this time slowly decreased with incubation time up to 30 min.

Effect of enzyme concentration. One can see in Fig. 3 that the rate of riboflavin formation was directly proportional to the enzyme concentration up to about 1 mg of protein equivalent. Augmentation of the protein level beyond this

Fig. 2. Time course of enzymatic formation of riboflavin. The enzymatic reaction was carried out in the medium and conditions described in the text. Enzyme equivalent to 1.4 mg of protein was used for the reaction. Riboflavin produced was determined at intervals as indicated above by the method described in the text.

content, however, resulted in reduction of the rate, which deviated far from linearity.

pH-dependency. The pH-activity curve of the enzyme is illustrated in Fig. 4. The enzyme exhibited an optimal activity at pH 6.5 with a broad plateau of pH ranges between 5.7 and 7.0. Reduction of the pH value lower than 5.7 caused an abrupt drop of the activity, and, also, the curve showed a gradual fall of activity in the alkaline region beyond pH 7.0. The activities observed at pH 5.0 and 8.0 were found to be 35.4% and 45.8% of the optimum respectively.

Fig. 3. Relationship between enzyme concentration and riboflavin formation. The enzyme activity was determined by the same procedure described in the text, except that the concentration of the enzyme used was varied as indicated above. Incubation was made for 20 min.

Fig. 4. pH-Activity curve of riboflavin synthetase. The same assay method was employed as described in the text, except that the pH of the reaction medium was varied, as indicated above; enzyme equivalent to 1.4 mg of protein was used. The reaction proceeded for 15 min. —□—, 0.2 M acetate buffer; —○—, 0.2 M phosphate buffer; and —△—, 0.2 M Tris-HCl buffer.

Effect of temperature. As shown in Fig. 5, the enzymatic reaction was markedly facilitated by the elevation of temperatures. Optimal activation was observed at 47°C. The reaction velocity dropped rapidly at temperatures higher than the optimal one. The enzyme was completely inactive at 60°C. Arrhenius plots of the logarithmic values of the reaction velocities against reciprocals of the absolute temperatures give a straight line as depicted in Fig. 6. An apparent activation energy of 11,000 calories per mole between 42° and 20°C was evaluated from the slope of the plot.

Fig. 5

Fig. 6

Fig. 5. Temperature-dependency of riboflavin synthetase activity. Enzyme activity was determined as described in the text, except that the reaction medium was incubated for 15 min at each temperature indicated above, enzyme equivalent to 1.1 mg of protein was used.

Fig. 6. Arrhenius plots of riboflavin synthetase. E , apparent activation energy; T , absolute temperature; and k , reaction rate constant.

Effect of substrate concentration. The effect of 6,7-dimethyl-8-ribityllumazine concentration change on the reaction velocity was investigated. The results presented in Fig. 7 show that the enzyme exhibited a sigmoidal kinetic response with increasing substrate concentrations (S). A plot of these data according to the method of LINEWEAVER and BURK (10) is found to give a curved plot and not a straight line, from which could be calculated a value for the maximum initial velocity (V) of approximately 2.3×10^3 units. A Hill plot of the initial velocities (v) consists of two straight lines with slope=2 at lumazine concentrations below 6×10^{-5} M and with slope=1 at the levels beyond this point, as shown in Fig. 8. On the other hand, on plotting $\log v/V$ against $\log S$, a curve was obtained as illustrated in Fig. 9, which consists of a straight line with slope=2 at the substrate concentrations below 2.5×10^{-5} M and a curve at the levels higher than this point. The tangent line on the curve at 1.5×10^{-4} M of the lumazine level is found to have a slope of 0.27 instead of 1, and also found not to intersect the $\log S$ axis at 0.

Fig. 7. Effect of concentration of 6,7-dimethyl-8-ribityllumazine on riboflavin synthetase reaction. The reaction velocity was determined in the media and conditions described in the text, except that the incubation was made for 15 min in a media containing enzyme equivalent to 1.1 mg protein and various amounts of 6,7-dimethyl-8-ribityllumazine as indicated above. v , initial velocity; and S , substrate concentration.

Fig. 8

Fig. 9

Fig. 8. Hill plot of the initial velocity of riboflavin synthetase reaction. v , V , and S denote initial velocity, initial maximum velocity and concentration of 6,7-dimethyl-8-ribityllumazine, respectively.

Fig. 9. A logarithmic plot of the initial rate of riboflavin synthetase reaction. v , V and S denote initial velocity, initial maximum velocity and concentration of 6,7-dimethyl-8-ribityllumazine, respectively.

Two possible models for the reaction mechanism catalyzed by riboflavin synthetase are conceivable. One involves a ternary enzyme-substrate complex as proposed by HARVEY and PLAUT (1) for yeast enzyme, from the results on fluorescent polarization of the binary complex in the presence of the free substrate. The other model contains a stage whereby the substrate acts on the binary complex without a significant formation of the ternary complex, as suggested by THEORELL and CHANCE (11) for liver alcohol dehydrogenase and by CHANCE for peroxidase (12).

The first model can be written as:

where E , S , Sa and Sd are the enzyme, 6,7-dimethyl-8-ribityllumazine, the lumazine bound to the acceptor site on the enzyme, and the lumazine bound to the donor site on the enzyme, respectively, and Ka , Kd , Ka' and Kd' are the respective dissociation constants of the equilibria indicated above, and $Ka'Kd = KaKd'$. The following rate equation can be formulated, assuming that all combinations with the enzyme have a rapid equilibrium rate compared with the rate of formation of the products:

$$\frac{1}{v} = \frac{1}{V} \left[1 + \frac{Ka'}{[S]} + \frac{Kd'}{[S]} + \frac{Ka'Kd}{[S]^2} \right]. \quad (2)$$

The logarithmic form of this equations is

$$\log \frac{v}{V-v} = 2 \log [S] - \log [(S)(Ka' + Kd') + Ka'Kd], \quad (3)$$

where v and V are the initial and maximal initial velocities, respectively. If $\log v/(V-v)$ is plotted against $\log [S]$ (Hill plot), a curve with two asymptotic straight lines with slope=2 at $[S] \ll Ka'Kd/(Ka' + Kd')$ and a slope=1 at $[S] \gg Ka'Kd/(Ka' + Kd')$ should be obtained. The former and latter asymptotes will cut the $\log [S]$ axis at $\log \sqrt{Ka'Kd}$ ($= \log \sqrt{KaKd'}$) and $\log (Ka' + Kd')$, respectively. These relationships have been analyzed in full in the previous report (2). This theoretical model was satisfactorily consistent with the kinetical data of the Hill plot for the *E. ashbyii* enzyme obtained at lumazine concentrations from 1.5×10^{-4} M to 6.3×10^{-6} M, as shown in Fig. 8. Values of $Ka'Kd$ (or $KaKd'$) and $Ka' + Kd'$ were calculated to be 2.7×10^{-9} M² and 4.5×10^{-5} M, respectively, from the intercepts of the two straight lines on the $\log [S]$ axis as shown in Fig. 8. If an ordered binding of the lumazine to the enzyme occurs in the ternary complex formation instead of the random mechanism mentioned above, two separate processes are possible as follows:

or

The velocity equations corresponding to these are revealed in the respective logarithmic forms as:

$$\log \frac{v}{V-v} = 2 \log [S] - \log [[S]Kd' + KaKd'] \quad (6)$$

or

$$\log \frac{v}{V-v} = 2 \log [S] - \log [[S]Ka' + Ka'Kd]. \quad (7)$$

It is certain from these relations that the intercept on the $\log [S]$ axis of the asymptote with a slope=1 in the Hill plot should correspond to Kd' or Ka' . Thus, Ka' and Kd (or Kd' and Ka) were evaluated as 4.5×10^{-5} M and 6.0×10^{-5} M, respectively, since $Ka'Kd$ (or $KaKd'$) = 2.7×10^{-9} M² as calculated previously.

The second model for riboflavin synthetase reaction can be written:

where K denotes a dissociation constant of the enzyme-substrate complex, ES . An assumption that the reaction rate is proportional to the ES concentration leads to

$$\frac{1}{v} = \frac{1}{V} \left[\frac{1}{[S]} + \frac{K}{[S]^2} \right]. \quad (9)$$

The logarithmic form of this equation is

$$\log \frac{v}{V} = 2 \log [S] - \log [[S] + K]. \quad (10)$$

On plotting $\log v/V$ against $\log [S]$, a curved line should be obtained with two straight asymptotes with a slope=2 for $[S] \ll K$ and a slope=1 at $[S] \gg K$. The former and latter straight lines will cut the $\log [S]$ axis at $\log \sqrt{K}$ and 0, respectively. The kinetical data presented in Fig. 9, however, does not agree with this theoretical model.

3. Purification of riboflavin synthetase

Step 1: Extraction. Operations in all steps of purification were carried out at 0 to 4°C, unless otherwise stated. Mycelial paste (868 g) of *E. ashbyii* har-

vested after 2 days' culture was mixed with alumina (1,300 g) of bacterial grade and ground for 10 min in the same fashion described previously under "EXPERIMENTAL." The resulting pastes were homogenized in 0.1 M potassium phosphate buffer (2.2 liter) with a pH 6.5 containing 10 mM mercaptoethanol, 10 mM Na_2SO_3 and 5 mM ethylenediaminetetraacetate (EDTA) sodium salts (pH 7.0) (Buffer A). The homogenate was centrifuged at 10,000 rpm for 10 min. The supernatant liquid (2,410 ml) was retained.

Step 2: Acidification. The extract was adjusted to a pH 5.5 under overhead stirring by a slow drop-by-drop addition of 3 M acetic acid, and allowed to stand overnight. The precipitate was removed by centrifugation. The supernatant solution was neutralized to a pH 6.5 with 1 N NaOH.

Step 3: First ammonium sulfate fractionation. Solid ammonium sulfate (978 g) was slowly added with stirring to the supernatant (2,500 ml) to give a final concentration of 60%. Stirring was continued for 2 hr. The pH of the solution was kept to a pH 6.5 by drop-by-drop addition of 1 N NaOH during this operation. The precipitate was collected by centrifugation and dissolved in the Buffer A.

Step 4: Second ammonium sulfate fractionation. The solution (500 ml) from Step 3 was diluted with Buffer A to give a protein concentration equivalent to 10 mg per ml (666 ml), and saturated to 38% by addition of solid ammonium sulfate (153 g) in the same manner as in Step 3. The suspension was centrifuged. To the supernatant liquid (720 ml), an additional solid ammonium sulfate (94.2 g) was added to bring the solution up to 58% saturation; this was followed by centrifugation. The precipitate was dissolved in Buffer A.

Step 5: Freezing-thawing treatment. The solution (400 ml) was slowly acidified to a pH 5.5 with 3 M acetic acid, followed by addition of mercaptoethanol (6.27 g), the final content of which was adjusted to 0.2 M. The solution was allowed to stand overnight under a frozen state at -20°C , and then it was

Table 2. Purification of riboflavin synthetase from *Eremothecium ashbyii*.

868 g of wet mycelia were submitted for purification of the enzyme. Step 1, extraction; Step 2, acidification; Step 3, first ammonium sulfate fractionation (0–0.6 saturation); Step 4, second ammonium sulfate fractionation (0.38–0.58 saturation); and Step 5, acidification and freezing-thawing treatment. The assay method of riboflavin synthetase was the same as that described in the text.

Step	Total volume (ml)	Total protein (mg)	Total activity ($\times 10^3$ unit)	Specific activity (unit per mg protein)	Recovery of activity (%)	Purification degree
1	2,410	17,700	2,920	165	100	1
2	2,500	12,000	3,090	257	110	1.6
3	500	6,660	2,840	427	100	2.6
4	400	4,200	2,270	541	78	3.3
5	28	176	1,120	6,370	38	39

thawed in water bath at 10°C. The resulting precipitate was removed by centrifugation. The supernatant (390 ml) was saturated to 60% by addition of solid ammonium sulfate (152 g) according to a procedure similar to that in *Step 3*. The precipitate recovered by centrifugation was dissolved in a small amount of Buffer A. The enzyme solution (28 ml) was kept at 4°C. The final preparation exhibited a specific activity of 6,370 units per mg protein, approximately purified 39-fold from the original extract, and the yield of the enzyme was about 38% of the content of the extract. A summary of the purification is given in Table 2.

DISCUSSION

In a previous report on a riboflavinogenic *E. ashbyii*, the present authors (5) have suggested that the genetic processes of transcription and translation for synthesizing the enzymes comprising the riboflavinogenic pathway may be limited to taking place within a confined period in the fungal life cycle, which is located in an earlier stage of the growth phase, and that the enzymes synthesized remain stable to function in the vitamin overproduction period which begins just in coincidence with the growth cessation. This suggestion is supplemented with powerful support from the present investigation.

As shown in Fig. 1, riboflavin synthetase catalyzing the final step in the vitamin synthesis exists with the highest intracellular level in the early period of the growth phase and persists throughout the life cycle of *E. ashbyii*, although the level drops to about 60 to 50% of the initial value after the vitamin overproduction is initiated. Furthermore, the enzyme level in this fungus is abnormally high when compared with those in riboflavin-forming microbes such as *Aerobacter aerogenes*, *Pseudomonas*, *Bacillus subtilis*, *Escherichia coli*, *Ashbya gossypii*, *Saccharomyces cerevisiae*, *Neurospora crassa*, and *Lactobacillus plantarum*. The specific enzyme activities detectable in the extracts from these microorganisms have been found to range from 0.6 to 1.6 mμmoles riboflavin produced per mg protein per hour at 37°C (13), whereas the extract of *E. ashbyii* shows an activity between 72 to 165 mμmoles of the vitamin formed, approximately 100 times higher than the former values, as shown in Fig. 1, and Tables 1 and 2. From this evidence, it seems plausible that synthesis of the riboflavinogenic enzymes including riboflavin synthetase might be controlled more loosely in *E. ashbyii* than in the other microbes. This implies that there is an essential difference between *E. ashbyii* and the other organisms at a regulatory gene or at an operator region of a riboflavin operon.

WILSON and PARDEE (14) studied vitamin synthesis in *E. coli* and reached a conclusion that repression rather than feedback inhibition can account for the mechanisms of vitamin production which is kept under a very low rate in normal conditions. It has been known that *A. gossypii* yields prosperously the vitamin only in a narrow range of temperature between 26°C and 28°C, despite its growth

over a wide range (15, 16). KAPLAN and DEMAINE (17) observed that the washed cell-suspensions prepared from the mycelia grown at 28°C are able to produce riboflavin at 37°C as well as at 28°C, whereas the mycelia cultured at 37°C are lacking in the riboflavinogenic ability at either temperature. On the basis of these findings, DEMAINE (18) has recently suggested that overproduction by *A. gossypii* is controlled by a repressor which fails to be formed at low temperature. This concept suggests a possibility that the repression mechanism might alter in *E. ashbyii* so as to cause an abnormal elevation in the intracellular levels of riboflavinogenic enzymes, which acts as one of the driving forces to induce vitamin overproduction. The derepression might happen by alteration in the function of the regulator gene or of the operator of the riboflavin operon as follows: (a) the repressor is not produced from the regulator gene, (b) the repressor formed has no potency to bind to the operator, (c) the repressor has a low affinity for the operator, (d) the operator loses affinity for the repressor, and (e) the affinity of the operator for the repressor is diminished.

It has been observed with riboflavin synthetase from spinach (3), baker's yeast (1) and *A. gossypii* (4) that Lineweaver-Burk plotting of the reaction velocity gives a straight line with a Michaelis constant in each case over a range of 6,7-dimethyl-8-ribityllumazine concentrations between 2×10^{-4} to 6×10^{-7} M. In contrast to this, the preceding investigation (2) with the enzyme of *E. ashbyii* demonstrated that the same plotting results in a curve instead of a straight line, and provided a suggestion that two lumazine molecules can bind to *E. ashbyii* enzyme with affinities of similar orders of magnitude, whereas there is a marked distinction between these affinities in the case of the other enzymes. In the latter case, the second order term, $Ka'Kd/[S]^2$, in the rate equation as indicated in Equation (2) makes a negligible contribution to $1/v$, and thus $Ka'Kd$ (or $KaKd'$) is restricted to the values lower than $3.6 \times 10^{-13} \text{ M}^2$, and the Michaelis constant obtained from the Lineweaver-Burk plot corresponds to the sum of the dissociation

Table 3. Comparison of catalytic properties of riboflavin synthetase from *E. ashbyii*, spinach, yeast and *A. gossypii*.

	<i>E. ashbyii</i> ^a	Spinach ^b	Yeast ^c	<i>A. gossypii</i> ^c
As a random mechanism ^e				
$Ka' + Kd'$ (K_m) (M)	4.5×10^{-5}	4.5×10^{-5}	4.0×10^{-5}	2.9×10^{-5}
$Ka'Kd$ (or $KaKd'$) (M^2)	2.7×10^{-9}	$< 3.6 \times 10^{-13}$	$< 3.6 \times 10^{-13}$	$< 3.6 \times 10^{-13}$ ^d
As a ordered mechanism ^e				
Ka' (or Kd') (M)	4.5×10^{-5}	4.5×10^{-5}	4.0×10^{-5}	2.9×10^{-5}
Kd (or Ka) (M)	6.0×10^{-5}	$< 8.0 \times 10^{-9}$	$< 9.0 \times 10^{-9}$	$< 1.2 \times 10^{-9}$
Optimum pH	6.5	7.5	7.0-7.5	6.9
Activation energy (cal./mole)	11,000	15,000	10,000	—

^a The present report; ^b MITSUDA *et al.* (3); ^c PLAUT (4); ^d $3.6 \times 10^{-13} \text{ M}^2$ is calculated on the evidence that the enzyme activity can be determined with the substrate concentrations beyond 0.6 μM ; and ^e in the substrate binding to the enzyme.

constants of the ternary enzyme-substrate complex, *i.e.*, $Ka' + Kd'$. Values of $Ka' + Kd'$ and $Ka'Kd$ (or $KaKd'$) for *E. ashbyii* enzyme are determined by Hill plotting of the reaction velocity as illustrated in Fig. 8. These kinetical constants are compared with those of the other enzymes as shown in Table 3. Values of $Ka' + Kd'$ of *E. ashbyii* enzyme are similar to those of the enzymes from spinach, yeast and *A. gossypii*, but $Ka'Kd$ (or $KaKd'$) of the former enzyme is at least 7,500-fold higher than those found in the latter enzymes. Such a large disparity appears due to one of the two sites on the latter each enzyme binding to the substrate with an extremely high affinity.

Indeed, HARVEY and PLAUT (1) isolated a stable binary enzyme-substrate complex, in which the substrate is tightly bound at the donor site instead of the acceptor site on yeast enzyme, favouring an ordered addition of the substrate to form a ternary enzyme-substrate complex, *ESaSd*, by the pathway represented by Equation (5) (*via ESd*). Also, an ordered mechanism seems applicable for *A. gossypii* and spinach enzymes, although it is not clear whether it proceeds through the pathway of Equation(4) or (5) (*via ESd* or *ESa*). On the other hand, however, it is not possible to distinguish between a random or ordered binding of the substrate for *E. ashbyii* enzyme, since the enzyme has two binding sites with similar affinities. If an ordered substrate binding occurs like yeast enzyme, the dissociation constants of the binary and ternary complexes, Kd and Ka' (or Ka and Kd'), can be calculated and compared for the respective enzymes as given in Table 3. In this case, the Michaelis constant corresponds to Ka' (or Kd'), which does not show a distinct variation among the enzymes of *E. ashbyii*, spinach, yeast and *A. gossypii*, giving 2.9×10^{-5} M to 4.5×10^{-5} M. Kd (or Ka) for *E. ashbyii* enzyme is evaluated as 6.0×10^{-5} M, which is comparable to Kd' (or Ka') of 4.5×10^{-5} M. In contrast, the other enzymes have Kd (or Ka) values lower than at least about 10^{-8} M.

All these results mentioned above suggest that *E. ashbyii* enzyme differs markedly from the other enzymes with respect to its affinity for the substrate and fundamental protein conformation at or around one of the two binding sites within the active center, possibly at the donor site rather than the acceptor site. The Lineweaver-Burk plot could give a curve which passes through 0 on both the horizontal and vertical axes, when a binary enzyme-substrate complex reacts directly with a free substrate without formation of a ternary complex (Equation 8). This mechanism, however, is not the case for *E. ashbyii* as shown in the present investigation. The apparent activation energy and pH optimum for the activity of *E. ashbyii* enzyme are compared with those of the enzymes of spinach, yeast and *A. gossypii* as represented in Table 3. There is no remarkable difference in these values, although *E. ashbyii* enzyme exhibits a significantly lower value of the pH optimum, pH 6.5, compared with the other enzymes, pH 7.0 to 7.5.

Riboflavin synthetase is difficult to isolate from living organisms, since its level is markedly low in extracts of organisms, as shown by tests (13). Recently,

PLAUT *et al.* (19) have purified the enzyme 3,860-fold from the cell-free extract of yeast by an improved procedure involving fourteen steps of purification. The final preparation was judged to show a single protein band by disc electrophoresis, which has a specific activity of 13,900 μ moles of riboflavin formed for one hour (13,900 units) per mg of protein at 37°C. The present report demonstrates that an extremely high content of the synthetase is present in *E. ashbyii*, and that it can be purified about 40-fold with a yield of 38% from the extract by the simplified method shown in Table 2, in which Step 5, a freezing followed by a thawing treatment of the enzyme in the presence of mercaptoethanol, is especially effective for raising the purity. The final sample obtained exhibits a specific activity of 6,370 units per mg of protein at 37°C. If the *E. ashbyii* enzyme has similar orders of magnitude to yeast enzyme with respect to its turnover number and molecular weight, the purity of the preparation is calculated to be about 46% against the sample containing only the synthetase. Further investigation will be needed to purify and characterize the *E. ashbyii* enzyme.

REFERENCES

- 1) HARVEY, R. A. and PLAUT, G. W. E., *J. Biol. Chem.*, **241**, 2120 (1966).
- 2) SUZUKI, Y. and MITSUDA, H., *Biochim. Biophys. Acta*, **242**, 500 (1971).
- 3) MITSUDA, H., KAWAI, F., SUZUKI, Y. and YOSHIMOTO, S., *J. Vitaminol.*, **16**, 285 (1970).
- 4) PLAUT, G. W. E., *J. Biol. Chem.*, **238**, 2225 (1963).
- 5) MITSUDA, H. and SUZUKI, Y., *J. Vitaminol.*, **16**, 172 (1970).
- 6) LOWRY, O. M., ROSENBROUGH, N. J., FARR, A. L., and RANDALL, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
- 7) KALCKAR, H. M., *J. Biol. Chem.*, **167**, 461 (1947).
- 8) GOODWIN, T. W., *The Biosynthesis of Vitamins and Related Compounds*, Academic Press Inc., New York and London, p. 24 (1963).
- 9) KAPRALEK, F., *J. Gen. Microbiol.*, **29**, 403 (1962).
- 10) LINEWEAVER, H. and BURK, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
- 11) THEORELL, H. and CHANCE, B., *Acta Chem. Scand.*, **5**, 1127 (1951).
- 12) CHANCE, B., *J. Biol. Chem.*, **151**, 553 (1943).
- 13) PLAUT, G. W. E., in D. M. Greenberg (Editor), *Metabolic Pathway*, Academic Press Inc., New York and London, Vol. II, p. 692 (1961).
- 14) WILSON, A. C. and PARDEE, A. B., *J. Gen. Microbiol.*, **28**, 283 (1962).
- 15) TANNER, F. W., Jr., VOJNOVICH, C. and VAN LANEN, J. M., *J. Bacteriol.*, **58**, 737 (1949).
- 16) PFEIFER, V. F., TANNER, F. W., Jr., VOJNOVICH, C., and TRANFLER, D. H., *Ind. Eng. Chem.*, **42**, 1776 (1950).
- 17) KAPLAN, L. and DEMAINE, A. L., in D. G. Ahearn (Editor), *Recent Trends in Yeast Research*, Georgia State Univ., Atlanta, p. 137 (1970).
- 18) DEMAINE, A. L., *Annual Review of Microbiology*, **26**, 369 (1972).
- 19) PLAUT, G. W. E., BEACH, R. L., and AOGAICHI, T., *Biochemistry*, **9**, 771 (1970).