GUANOSINE NUCLEOTIDE PRECURSOR FOR FLAVINOGENESIS OF *EREMO- THECIUM ASHBYII*¹

Hisateru MITSUDA and Kenji NAKAJIMA²

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

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Summary The purine precursor in the riboflavin biosynthetic pathway in *Eremothecium ashbyii* was examined using a guanine analogue, 8-azaguanine, with non-growing cell systems.

1. Riboflavin formation in the culture filtrate was determined at 0, 5, 10 and 20 hr after start of the incubation of the non-growing cells in the presence of xanthine or 8-azaguanine (1 mM, respectively). At 20 hr of incubation, the addition of xanthine stimulated riboflavin formation by 36% and the addition of 8-azaguanine inhibited the formation by 57%.

2. Acid soluble nucleotide pools in the cells were followed at 0, 5, 10 and 20 hr of the incubation period in the presence of xanthine or 8-azaguanine by means of anion exchange column chromatography. The result showed that the GTP pool changed markedly despite the fact that the adenosine nucleotide pool was almost constant irrespective of the presence or absence of these purines till 10 hr of incubation. But, the decrease of the former was overcome in part by the addition of flavinogenic xanthine. Furthermore, the total amounts of GTP and guanosine accumulated in cells in the presence of 8-azaguanine reached the maximum already at 5 hr, attaining a level twice as much as the GTP contents of the control.

3. The role of guanosine nucleotide pool in riboflavin formation was further examined using 8-azaguanine. In this experiment the drug was added to the suspension of non-growing cells at 3 hr or 6 hr after the incubation was started and the reaction was continued till the 12th hr. A more clear-cut correlation between riboflavin formation and guanosine nucleotide pool was observed by this experiment. The guanosine nucleotide pool (consisting of GMP, GDP and GTP) increased simultaneously with the inhibition of riboflavin formation. Of the guanosine nucleotides pools, the GMP pool increased 2.7 times above normal upon

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² 満田久輝, 中島謙二

the addition of 8-azaguanine during the incubation for 6 hr and 5.3 fold for 9 hr. While, the GTP pool increased 1.9 fold above normal for 6 hrs' incubation in the supplementation of this drug but decreased to one-half of the normal at the incubation period of 9 hr. In these cases, the decreased amounts of GTP were equal to the increased amounts of GMP during the incubation periods of 6 hr and 9 hr in the presence of added 8-azaguanine.

4. The above results suggest strongly that GTP is an immediate precursor of riboflavin in the form of nucleotide.

It has been well established that purines are precursors of riboflavin biosynthesis and the C₍₈₎ of the imidazole ring of purine base is discharged on a certain step of flavinogenesis by work with isotopes (1-6).

Furthermore, there are many reports (2, 5, 7, 8) that guanine and xanthine among various purine bases are the most efficient stimulants for the biosynthesis. The present authors (9, 10) also reported that guanine and xanthine, especially the latter noticeably stimulates riboflavin biosynthesis of *Eremothecium ashbyii*. Furthermore, in non-growing cells of *E. ashbyii* (11), the present authors obtained the results (12, 13) showing that riboflavin biosynthesis may proceed through GMP resulting from highly flavinogenic xanthine and the possibility (14) that guanosine triphosphate is an immediate precursor at the level of purine nucleotide.

In the present paper, we further investigated the possibility of guanosine triphosphate as a precursor in the riboflavin biosynthetic pathway, using a guanine analogue, 8-azaguanine in the non-growing cell systems of *E. ashbyii*.

METHODS

Eremothecium ashbyii IFO 0944 (flavinogenic strain) was obtained through the courtesy of the Institute for Fermentation, Osaka. The mycelia were cultivated for 1 day with a rotary shaker (220 rpm) on a natural nutrient medium (pH 5.5) consisting of 2% glucose, 1% peptone, 0.3% yeast extracts, 1.8% Tween 80 and mineral salts (9). Following this experiments with non-growing cells of *E. ashbyii* (11) were carried as follows.

The mycelia cultivated for 1 day (at the exponential phase) were harvested by filtration from the natural nutrient medium and adequately washed with distilled water. The mycelia were suspended in 0.1 M phosphate buffer (pH 6.8) containing 0.5% glucose at the ratio of 20 ml buffer per 1 g mycelia (wet weight). The mycelial suspension was starved by incubating at 27°C for 8 hr in the dark on a reciprocal shaker (frequencies 62/min). After starvation, the mycelia were collected, washed and resuspended in the same buffer containing the test compounds. The compounds were infiltrated into mycelia under reduced pressure in a desiccator at intervals of 2 min for 2, 3 and 5 min in this order. The suspension was incubated

on a reciprocal shaker (frequencies 85/min) by the same methods as those of starvation. The mycelia after the incubation were used for the extraction of nucleotides.

Perchloric acid (P. C. A.) extraction (13) of cells was carried out by mixing and grinding cells (10 g wet weights) in a mortar with distilled water (20 ml) and 3 M perchloric acid solution (4 ml). The mycelial slurry was centrifuged. The supernatant solution was adjusted to pH 7.0 with 3 M potassium hydroxide solution and allowed to stand for 15 min. The clear solution obtained by decantation was used in the following experiments. All the procedures mentioned above were carried out in the cold (4°C).

Ten milliliters of the nucleotide extracts were loaded on a column (0.7 × 30 cm) of Dowex 1 × 2 (formate, 200–400 mesh). The column was developed by a stepwisegradient elution system (13) in which the following solvents flow successively into a mixing chamber containing 50 ml of distilled water: distilled water (40 ml) for washing at the flow rate of 1.8 ml/5 min; then, (I) 0.5 M formic acid (100 ml), (II) 2 M formic acid (100 ml), (III) 2.5 M formic acid-0.18 M ammonium formate (100 ml), (IV) 3 M formic acid-0.31 M ammonium formate (150 ml), and (V) 3 M formic acid-2.2 M ammonium formate (100 ml) at the flow rate of 0.9 ml/5 min, respectively. The absorbance of the eluate from the column was automatically determined at 253.7 nm by means of LKS UVICORD (4701A Type) having a light path of 0.3 cm. Each nucleotide content was determined from the volume of the eluting solution and the mean optical densities using the respective molar extinction coefficients (15). The compounds accumulated by the addition of 8-azaguanine (the fractions containing them are indicated by shadowing in figures) have already been identified as uridine, 8-azaguanine, guanosine, 8-azaguanosine and 8-azaguanosine triphosphate in the order of the elution from the column (14).

Flavins in figures were determined from the absorption values at 450 nm of samples by using 12.2 at 450 nm as the millimolar extinction coefficient of riboflavin.

RESULTS

1. *Effects of 8-azaguanine and xanthine on riboflavin formation during non-growing cell incubation*

Effects of 8-azaguanine and xanthine on riboflavin formation in non-growing cell culture of *E. ashbyii* were examined by the addition of the drug at the start of the incubation at 10^{-3} M and the results are shown in Fig. 1. As many experiments have shown that riboflavin formation in the medium is in proportion to that in cells, only riboflavin formation of the medium was determined in this experiment.

As shown in the figure, riboflavin was exponentially produced with incubation time in these cases. Xanthine effectively stimulated riboflavin formation as reported previously (9, 10, 12). On the other hand, 8-azaguanine inhibited formation. The inhibitory effect enhanced linearly with the incubation time. At 20 hr

Fig. 1. Effects of xanthine and 8-azaguanine on riboflavin production of the non-growing cell medium. These purines were added to the medium at the start of the incubation at 10^{-3} M. After the incubation of non-growing cell for the indicated time, the mycelial suspensions were filtered through filter paper and the absorbances at 450 nm of the filtrate were determined. For details of this experiment see METHODS.

of incubation, xanthine stimulated riboflavin formation by 36% and 8-azaguanine inhibited the formation by 57% as compared with the control.

2. *Fluctuation of acid soluble nucleotides in non-growing cells in the presence of 8-azaguanine and xanthine*

The acid soluble nucleotides in the cells under the same conditions as described above were examined by means of anion-exchange column chromatography. As shown in Figs. 2, 3 and 4, the elution patterns of nucleotides in the case of the control and of the xanthine addition showed a similar pattern during the incubation. In both cases, adenosine nucleotide pool contained little amounts of AMP and large amounts of ADP and ATP, whereas guanosine nucleotide pool contained traces of GMP and GDP and large amounts of GTP as observed on these chromatograms. But, in the addition of 8-azaguanine, peaks of uridine (a peak before that of 8-azaguanine), 8-azaguanine (8-azaG), guanosine (GR), 8-azaguanosine (8-azaGR) and 8-azaguanosine triphosphate (8-azaGTP) in this order were ascertained even at 5 hr of incubation.

From these results, the contents of adenosine and guanosine nucleotides being main peaks on these chromatograms were calculated by the procedures described

Fig. 2. Acid soluble nucleotide pools in cells at 0 and 5 hr after incubation of non-growing cells in the presence of xanthine or 8-azaguanine. These purines were added to the medium at the start of incubation at 10^{-3} M. After the non-growing cell incubation for 0 and 5 hr, the mycelia were harvested, washed and extracted with perchloric acid solution. Ten ml of the extracts were applied to a column packed with Dowex 1×2 resin (formate) and the absorbances at 253.7 nm were automatically recorded. See the details in the METHODS for the experimental procedures. Abbreviations: 8-azaG, 8-azaguanine; GR, guanosine; 8-azaGR, 8-azaguanosine; 8-azaGTP, 8-azaguanosine triphosphate.

in METHODS. The results are shown in Tables 1 and 2. The values in the tables indicate the relative amounts of adenosine and guanosine nucleotides. The relative ratios shown in parentheses are the amounts at which adenosine monoester were expressed as 1. Furthermore, nucleotides were expressed as the amounts per 10 ml of a P. C. A. extract which contained cells of about 2.6 g wet weights.

Fig. 3. Acid soluble nucleotide pools in cells at 10 hr incubation in the presence of xanthine or 8-azaguanine (10^{-3} M). The experimental conditions are the same as those in Fig. 2.

Adenosine nucleotide pool shown as AMP+ADP+ATP in this table is almost unchangeable till 10 hr of incubation. The situation is also similar for ATP. On the other hand, at 20 hr of incubation when riboflavin was markedly formed, both the pool of adenosine nucleotides and the amount of ATP varied to a fair extent in these cases. It is of great interest that the addition of xanthine during this incuba-

tion time brought about a noticeable increase of ADP and ATP contents and as a result, the increase of adenosine nucleotide pool. Also, the addition of 8-azaguanine resulted in the increase of ADP and ATP contents and adenosine nucleotide pool during the incubation.

While, as seen in Table 2, the content of GTP seems to decrease with the for-

Table 1. Relative amounts of adenosine nucleotide in non-growing cells at various incubation times.

These amounts were calculated from the results of Figs. 2, 3 and 4 and expressed as amounts per 10 ml P. C. A. extracts. The numbers in parentheses indicate the relative ratios of ADP and ATP at which AMP contents were expressed as 1 under the same experimental conditions.

Time	Addition	AMP	ADP	ATP	AMP+ADP +ATP
0 hr		0.07 (1)	μ moles 0.36 (1.9)	1.82 (9.5)	2.26
5 hr	None	0.03 (1)	0.27 (7.9)	1.86 (54.8)	2.17
	8-Azaguanine	0.03 ^a (1)	0.31 (9.2)	1.97 (58.0)	2.32
	Xanthine	0.05 (1)	0.28 (5.9)	1.76 (37.4)	2.08
10 hr	None	0.03 (1)	0.17 (5.0)	1.80 (54.7)	2.01
	8-Azaguanine	0.03 ^a (1)	0.28 (8.2)	2.22 (65.3)	2.53
	Xanthine	0.06 (1)	0.30 (5.2)	1.88 (31.9)	2.25
20 hr	None	0.05 (1)	0.12 (2.7)	0.53 (11.6)	0.70
	8-Azaguanine	0.05 ^a (1)	0.27 (5.8)	0.99 (21.5)	1.30
	Xanthine	0.05 (1)	0.36 (7.2)	1.19 (23.9)	1.60

^a AMP contents of None at the same time were shown because AMP contents in these cases could not exactly calculated due to the overlap of the neighbouring peak (GR). The method of the calculation is described in the METHODS.

Table 2. Relative amounts of guanosine nucleotide in non-growing cells at various incubation times.

These amounts were calculated from the results of Figs. 2, 3 and 4 and expressed as amounts per 10 ml P. C. A. extracts.

Time	Addition	GR	8-azaGR	GTP	8-azaGTP	GR+8- azaGR+ GTP+8- azaGTP
0 hr				μ moles 1.16		
5 hr	None			0.68		
	8-Azaguanine	0.61	0.16	0.69	0.12	1.58
	Xanthine			0.91		
10 hr	None			0.70		
	8-Azaguanine	0.54	0.16	0.82	0.27	1.77
	Xanthine			0.77		
20 hr	None			0.49		
	8-Azaguanine	0.41	0.22	0.54	0.08	1.24
	Xanthine			0.78		

Abbreviations; GR, guanosine; 8-azaGR, 8-azaguanosine; 8-azaGTP, 8-azaguanosine triphosphate.

Fig. 5. Relative ratios of guanosine and guanosine nucleotides against GTP amounts of control at various incubation times. The values of Table 2 were replotted in this figure.

mation of riboflavin. This fact is especially, remarkable during the incubation time from 0 to 5 hr, which was not found in the adenosine nucleotide pool shown in Table 1. The same attitude as that concerning GTP was also observed in 8-azaGTP pool in the addition of 8-azaguanine. However, in this case, the decrease of 8-azaGTP possibly correlates with the increase of 8-azaguanosine pool. It is noteworthy that such a change in the 8-azaGTP pool was observed under the inhibition of riboflavin formation in the presence of 8-azaguanine.

Furthermore, the results obtained in Table 2 are depicted again in Fig. 5 in regard to only the changes of guanosine and guanosine nucleotides to clarify the inhibitory mechanism of 8-azaguanine on the riboflavin biosynthetic pathway. Figure 5 shows the relative amounts of guanosine and guanosine nucleotides compared with the GTP amount of the control at various incubation times.

The behaviours of GTP in the addition of xanthine and 8-azaguanine were peculiar. At 10 hr of incubation in the addition of 8-azaguanine, the amount of GTP was 1.2 times as much as that of the control. While, at 20 hr in the presence of xanthine when riboflavin formation markedly proceeds, the GTP compared to the control content increased 1.6 fold. It is further interesting that, in the presence of 8-azaguanine, the sum of guanosine and GTP contents, native purine derivatives in cells, was equivalent to about 2 times as much as the control GTP. At this time, the amounts of riboflavin were less than one-half of those of the control.

Fig. 6. Effects of 8-azaguanine on acid soluble nucleotide pools in non-growing cells. 8-Azaguanine (10^{-3} M) was added to the non-growing cell medium at 3 hr or 6 hr incubation and the reaction was stopped by grinding cells at 12 hr incubation. The experimental conditions of the column chromatography are the same as those in Fig. 2.

Furthermore, the total amounts of GTP, 8-azaGTP, guanosine and 8-azaguanosine in the presence of added 8-azaguanine were 2.5 times as much as the GTP content in the control. Total amounts of 8-azaguanosine and 8-azaGTP in this case may reflect the transformation activity of 8-azaguanine to corresponding nucleotides in this mold. Riboflavin formation to the addition of xanthine ap-

pears to have been stimulated through a similar metabolic pathway to that of 8-azaguanine, as seen in Fig. 1. The biosynthetic pathway from exogenous xanthine to riboflavin was clarified in the previous paper (12) referring to the reports of BERLIN (16) and MAGER (17).

3. *Effects of 8-azaguanine added at different incubation times on riboflavin formation and acid soluble nucleotide pools*

It was elucidated in Table 1 and Fig. 5 that guanosine and guanosine nucleotide increase noticeably till 10 hr in spite of the fact that the adenosine nucleotide pool remained unchanged even in the presence of 8-azaguanine. Since riboflavin is exponentially produced during this incubation, it is evident that riboflavin biosynthesis is closely correlated to the changes of guanosine nucleotide pool. Accordingly, the effects of 8-azaguanine on riboflavin formation and adenosine and guanosine nucleotide pools were further examined. In this experiment, 8-azaguanine was added at 3 hr or 6 hr after incubation was started and the reaction was stopped at 12 hr of incubation.

As seen in Fig. 6, these patterns are a little different from previous nucleotide patterns (Figs. 2, 3 and 4) in the point that guanosine and 8-azaguanosine triphosphate were not observed even by the addition of 8-azaguanine. However, it is to be noted in this experiment that guanosine mono-(GMP) and diphosphate (GDP), especially the former was clearly observed. This made it possible to pursue the fluctuation of guanosine nucleotides in connection with riboflavin formation. From this figure, each nucleotide content is calculated by the same method as that of Tables 1 and 2. The results are shown in Table 3.

Table 3. Relative amounts of adenosine and guanosine nucleotides at 12 hr incubation.

The amounts of these nucleotides were calculated from the results of Fig. 6. The numbers in parentheses show the relative ratios of ADP and ATP (or GDP and GTP) at which AMP (or GMP) contents were expressed as 1 under the same experimental conditions. For the method of calculation see METHODS.

	8-Azaguanine addition		
	None	at 3 hr	at 6 hr
	μ moles		
AMP	1.23 (1)	1.36 (1)	1.50 (1)
ADP	0.67 (0.54)	0.31 (0.23)	0.26 (0.17)
ATP	0.16 (0.13)	0.46 (0.34)	0.34 (0.21)
AMP+ADP+ATP	2.06	2.13	2.10
8-azaGR		0.14	
GMP	0.07 (1)	0.37 (1)	0.19 (1)
GDP	0.13 (1.86)	0.16 (0.44)	0.14 (1.02)
GTP	0.12 (1.72)	0.06 (0.17)	0.23 (1.67)
GMP+GDP+GTP	0.32	0.59	0.56

Fig. 7. Fluctuation of riboflavin formation and guanosine nucleotides in the presence of xanthine and 8-azaguanine. The results of Table 3 are replotted in this figure. Only the amounts of flavins are concerning those of the medium.

The table shows that the guanosine nucleotide pool remarkably changed, although the adenosine nucleotide pool was almost kept unchanged in the presence or absence of 8-azaguanine as seen in Tables 1 and 2. These results are illustrated in Fig. 7 in relation to riboflavin formation.

It is obvious from this table that riboflavin formation decreased in proportion to the period during which the cells were incubated with 8-azaguanine. Riboflavin formation was inhibited by 40% during the incubation with 8-azaguanine for 6 hr and by 55% for 9 hr as compared with control during 12 hr incubation.

On the other hand, total amounts of GMP, GDP and GTP increased by 75% for 6 hrs' incubation in the presence of the drug and by 84% for 9 hr. This indicates that the guanosine nucleotide pool is inversely proportional to riboflavin formation.

Furthermore, it is of great interest that there are singular properties in each guanosine nucleotide pool, especially in the GMP and GTP pools. The GMP pool linearly increased in proportion to the incubation period with 8-azaguanine,

while the GTP pool increased like the GMP pool by incubation with 8-azaguanine for 6 hr but markedly decreased during the 9 hr period, although the GDP pool remained almost constant during 12 hr incubation. Moreover, it should be noted in this figure that the increased amounts of GMP during a certain incubation period with 8-azaguanine was equal to the decreased amount of GTP in the same period: for example, the increase of GMP during an incubation period of from 6 hr to 9 hr was 0.07 μ mole and the decrease of GTP during the period was 0.065 μ mole, respectively.

DISCUSSION

It was verified in previous paper (14) that 8-azaguanine directly inhibits riboflavin formation under the experimental conditions using non-growing cells although it is well known to have effects on metabolic pathways (18, 19), especially purine metabolism (16, 20–23). In this experiment (14), marked accumulation of a number of 8-azaguanine derivatives in cells was observed. Especially, the accumulation of 8-azaguanosine triphosphate, not 8-azaguanosine mono- or diphosphate, in the cells suggests that a precursor of riboflavin may be guanosine triphosphate. Recently, it was reported that guanine or a guanine derivative may be a precursor of riboflavin by experiments using mutants lacking XMP aminase (24) and an antibiotic, decoyinine, to block the conversion of XMP to GMP (25).

The results presented here show that the addition of 8-azaguanine brings about a drastic change in the guanosine nucleotide pool despite the fact that the adenosine nucleotide pool is almost constant (see Tables 1, 2, 3 and Figs. 5, 7). In this case, the increasing rate of guanosine nucleotide pool (GTP+GR shown in Fig. 5) was almost equal to the decreasing rate of riboflavin formation (Fig. 1). Accordingly, it is readily seen that guanosine nucleotide pool has a close relation to flavinogenesis.

On the other hand, it is of great interest that total amounts of adenosine nucleotides (adenosine nucleotide pool) was maintained almost constant until the 12 hr incubation, in spite of the differences in the experimental conditions (Tables 1 and 3) or the physiological conditions of cells (Figs. 3 and 6). However, the localization of each adenosine nucleotide in the adenosine nucleotide pool was differently observed as seen in Figs. 3 and 6. The significance of the adenosine nucleotide pool and the localization of adenosine nucleotides in the pool was discussed in relation to flavinogenesis in growing cells of *E. ashbyii* (13). Also in non-growing cells of *E. ashbyii*, it seems possible that the localization takes part in the regulatory mechanism of the riboflavin biosynthesis. However, this problem remains obscure at present. Accordingly, the problem should be solved in detail in the future from the view point of regulation in flavinogenesis.

It is not known up to now whether any type of purine is a direct precursor

Fig. 8. Proposed pathway for flavinogenesis. Abbreviations: X, xanthine; 8-azaG, 8-azaguanine; GMP, guanosine monophosphate; GTP, guanosine triphosphate; TAP deriv., triaminopyrimidine derivative.

for flavinogenesis in the form of nucleotide. As for this problem, the present work provides interesting information—that is, addition of 8-azaguanine caused a marked increase in the GTP pool after 6 hr and a significant decrease in the pool after 9 hr accompanied by a stronger inhibition of flavinogenesis (Fig. 7). While, the GMP pool progressively increased in proportion to the incubation time with 8-azaguanine. In this case, the increased amount of GMP pool from 6 hr to 9 hr of the incubation with 8-azaguanine was equal to the decreased amount of GTP pool during the period.

These results imply the following processes; 8-azaguanine in cells inhibits GTP cleaving enzyme catalysing the opening at C₍₈₎ of the purine, although it is unclear whether this enzyme is the same as the enzyme taking part in the biosynthetic pathway of folic acid (26, 27). As a result, GTP being a precursor of riboflavin accumulates in cells by the inhibition of flavinogenesis for 6 hr, but thus accumulated GTP decreases to a great extent by the longer inhibition of flavinogenesis for 9 hr and is funnelled into the GMP pool, because of the maintaining of normality of the nucleotide pools in cells under these conditions.

Accordingly, the results obtained in the present paper strongly suggest that GTP is a direct precursor for flavinogenesis in the form of nucleotide. These results are further summarized in Fig. 8.

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