RELATIONSHIP BETWEEN ACCUMULATION OF GUANINE RIBONUCLEOTIDYL-(3’-5’)-ADENOSINE AND FORMATION OF RIBOFLAVIN

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Summary The possibility whether guanine ribonucleotidyl-(3’-5’)-adenosine (G₃A) is accumulated or not was studied with the use of microorganisms such as E. ashbyii (a non-flavinogenic strain), E. coli, S. cerevisiae and N. crassa, which produce riboflavin in a trace amount.

(1) In a flavinogenic strain of E. ashbyii, riboflavin formation was stimulated fivefold in the presence of glucose (1%) compared with that in the control experiment without glucose. The presence of caffeine noticeably restricted riboflavin formation during incubation of non-growing cells with or without glucose. Moreover, the addition of caffeine to the glucose-free medium brought about marked accumulation of G₃A in the cells.

(2) In a non-flavinogenic strain of E. ashbyii, riboflavin formation was remarkably slight under normal conditions. The effect of glucose and caffeine on flavinogenesis in the same strain was much smaller than that in the flavinogenic strain except for the case of the glucose-free medium supplemented with caffeine. However, compound (G₃A) never accumulated in the strain, even under conditions permitting a large amount accumulation of G₃A in a flavinogenic strain.

(3) The other organisms, E. coli, S. cerevisiae and N. crassa, did not accumulate G₃A in the cells under the same conditions as those with a high flavinogenic strain of E. ashbyii.

(4) The results obtained indicated that a dinucleotide, G₃A, is a compound closely related to the biosynthesis of riboflavin.

A close relationship exists between the formation of riboflavin and the fluctua-

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tion of nucleotide pools in flavinogenic microorganisms. Purine nucleotides have been known to be synthesized de novo (1), and to be incorporated into the skeleton of riboflavin except for C-8 of the purine ring (2-4).

This relationship has been extensively studied by experiments with various purines (5, 6) or methyl-xanthines (7) as reported previously. Caffeine strongly inhibited riboflavin formation by the flavinogenic strain of E. ashbyii (7). Then, accumulation of an unknown compound was observed in non-growing cells of the mold incubated in the glucose-free medium with caffeine. The compound proved to be guanine ribonucleotidyl-(3'-5')-adenosine (GpA) (8). The isolated dinucleotide, GpA, has never been found in animals, plants and microorganisms. Thus, the origin, the roles and the relation to flavinogenesis of this dinucleotide remain obscure.

At the same time, caffeine has been reported to induce genetic mutation by attacking the cellular DNA or RNA (9, 10). Accordingly, it is raised the problem of whether GpA is a degradation product of RNA or a substance possibly related to flavinogenesis.

In the present report, these problems were investigated with a flavinogenic and non-flavinogenic strains of E. ashbyii and other microorganisms including bacteria, yeast and fungus.

**EXPERIMENTALS**

*Materials.* Dowex 1 × 2 (Cl−) (200–400 mesh) and Dowex 50W × 4(H+) (100–200 mesh) were purchased from Dow Chemicals Ltd. All chemicals used were of the best quality available commercially.

*Organisms.* Eremothecium ashbyii strains, IFO 0944 (flavinogenic strain) and IFO 1425 (non-flavinogenic strain), were obtained from the Institute for Fermentation Osaka. The other microorganisms, Escherichia coli, Saccharomyces cerevisiae, and Neurospora crassa IFO 6178, were obtained through the courtesy of the Laboratory of Industrial Microbiology, Department of Food Science and Technology, Kyoto University. These microorganisms were maintained in the same way as described in previous papers (11, 12).

*Growing and non-growing cell experiments.* The cells of E. ashbyii, E. coli, S. cerevisiae and N. crassa, precultured on agar slants, were respectively suspended in 50 ml of inoculum medium in 300 ml Erlenmeyer flasks, and were allowed to grow under agitation for the following periods. After 24 hr of cultivation of E. ashbyii (the flavinogenic strain), E. coli, S. cerevisiae and N. crassa, and after 48 hr of cultivation of E. ashbyii (the non-flavinogenic strain), 5 ml amounts of the inoculum were inoculated into 500 ml of a basal medium in 2-liter Erlenmeyer flasks.

The basal medium (pH 5.5) contained 2% glucose, 1.8% Tween 80, 1% peptone, 0.3% yeast extract, 0.2% KH₂PO₄·2H₂O, 0.1% NaCl, 0.01% MgSO₄·7H₂O and distilled water. Growth was allowed to proceed in the dark with agitation for 20 hr.
in *E. coli* and *S. cerevisiae*, for 24 hr in *N. crassa* and *E. ashbyii* (the flavinogenic strain) for 48 hr in *E. ashbyii* (the non-flavinogenic strain).

At the end of the cultivation periods, the growing cells of *E. coli* and *S. cerevisiae* were collected and washed several times by centrifugation. In the meantime, the mycelia of *E. ashbyii* and *N. crassa* were collected by filtration and washed well with distilled water. The microorganisms obtained were suspended in 0.1 M phosphate buffer (pH 6.8) at a ratio of 20 ml buffer per 1 gram cells and incubated for 18 hr as described in the previous reports (13, 14) except that the starvation period was reduced to 4 hr in the case of the bacteria and the yeast. After the incubation, the cells of *E. coli*, and *S. cerevisiae* were subjected to column chromatographic analysis. While in the cases of both strains of *E. ashbyii*, a part of the mycelia was used for column chromatographic analysis and the remaining mycelia was used for measurement of riboflavin yields.

**Determination of riboflavin content in a flavinogenic and a non-flavinogenic strain of E. ashbyii.** For determination of riboflavin content, the incubation mixture of both strains was extracted by heating at 80°C for 10 min. The clear fluid obtained by centrifugation was submitted to determination of absorbance at 450 nm. The riboflavin content measured in this way was expressed as total flavins (μg/g wet-weight) in which the values of the medium and mycelia were given. Furthermore, for determination of riboflavin content in the mycelia of a non-flavinogenic strain, the mycelia was obtained by centrifugation of the incubation mixture and heated at 80°C for 10 min in distilled water (5 ml/g wet-mycelia). The mixture was centrifuged. The absorbance at 450 nm of the resulting clear supernatant was determined. The amounts of riboflavin were calculated using a millimolar extinction coefficient 11.2 at 450 nm and expressed as μg/g wet mycelia.

**Column chromatography on Dowex 1 × 2.** After being incubated for indicated time, the cells (10 g), were suspended in distilled water (20 ml) and 3 M perchloric acid (4 ml) was added to the suspension. The mycelial mixture was ground in a mortar for 10 min. The slurry was centrifuged and the resulting supernatant was adjusted to pH 7.0 with 3 M KOH. The resulting precipitate (KClO₃) was discarded by decantation, and the supernatant (10 ml) was applied to a Dowex 1 × 2 (Formate⁻) column (0.7 × 30 cm) which was washed with 40 ml of distilled water at a washing speed of 2.0 ml per 5 min. Chromatography was performed by means of an elution system in which 0.5 N formic acid flowed into a mixing chamber containing distilled water (50 ml) at a flow rate of 0.9 ml per 5 min (5).

**RESULTS**

(I) Effect of glucose and caffeine on riboflavin formation and nucleotide pools in a flavinogenic strain of *E. ashbyii*

Figure 1 shows the effect of caffeine and glucose on riboflavin biosynthesis during starvation and non-growing cell incubation. Riboflavin formation in the
Fig. 1. Effect of caffeine and glucose on riboflavin production by non-growing cells of a flavinogenic strain of \textit{E. ashbyii}. The mycelia were obtained after cultivation in 2-liter Erlenmeyer flasks containing 500 ml of a basal medium. The starvation and non-growing cell incubation were done in K-phosphate buffer in combinations of glucose and caffeine as shown below. At the end of the incubation period, total flavin was determined photometrically by reading absorbance at 450 nm. For experimental conditions see details in Experimentals.

(1) G(-)* G(-)** Caf(+)** (2) G(-)* G(-)** Caf(-)**
(3) G(+)* G(+)** Caf(+)** (4) G(+)* G(+)** Caf(-)**

G: glucose, Caf: caffeine, (+): presence, (-): absence
* at starvation, ** at non-growing cell incubation.

incubation for 18 hr on a glucose-free medium ((2) in Fig. 1) was only 20\% of that on a glucose-added medium (4). This indicates that glucose remarkably stimulated riboflavin formation in non-growing cells of the mold. Furthermore, supplementation of 5 mM caffeine in the glucose-free medium (1) brought about 40\% inhibition of the riboflavin formation compared to that of the control (2). The addition of 5 mM caffeine to the glucose-added medium (3) also inhibited riboflavin biosynthesis, although the inhibition was more markedly observed in the glucose-free medium during non-growing cell incubation. Furthermore, accumulation of G\textsubscript{p}A (8) was tested under the above experimental conditions and confirmation of the dinucleotide was carried out using an anion exchanger, Dowex 1\times2. The results are given in Fig. 2. An appreciable accumulation of G\textsubscript{p}A was found in the mycelia incubated in the glucose-free medium ((1) and (2) in Fig. 2). The accumulation of this compound increased on the addition of caffeine to the extent as indicated by the relations of (1) and (2), or (3) and (4). Moreover, the addition of caffeine, either to the glucose-free medium (2), or to the glucose supplemented medium (4), caused more marked accumulation of G\textsubscript{p}A.

Thus, the largest accumulation of G\textsubscript{p}A took place in the mycelia fed caffeine in the glucose-free medium. While, the riboflavin formation was lowest under these conditions.

Accordingly, these results indicate that the accumulation of G\textsubscript{p}A is closely related to riboflavin formation.

In the next sections, these phenomena were further studied with low riboflavin
Fig. 2. Effect of caffeine and glucose on nucleotide pools in non-growing cells of a flavinogenic strain of E. ashbyii. Acid soluble extract (10 ml) from the mycelia obtained under the conditions shown in Fig. 1 was placed on a column of Dowex 1×2 (formate-) which was followed by the gradient elution. Flow rate was 0.9 ml per 5 min. The absorbance of eluate was automatically recorded by the LKB uvicord (Type 4701). For experimental conditions see details in Experimentals.

(2) Effect of glucose and caffeine on riboflavin formation and nucleotide pools in a non-flavinogenic strain of E. ashbyii

The same experiments as those in Figs. 1 and 2 were carried out using the non-flavinogenic strain of E. ashbyii. Figure 3 shows the effect of the caffeine and glucose on riboflavin formation during non-growing cell incubation of the non-flavinogenic strain of E. ashbyii. Riboflavin formation (3.6 μg/ml) in the strain was remarkably less than that (118 μg/ml) of the flavinogenic strain under the same conditions. Furthermore, riboflavin formation in the mold was not stimulated by the addition of glucose ((2) and (4) in this figure.). The inhibition of riboflavin formation by caffeine occurred only in glucose free-medium. While, the change of riboflavin content in mycelia became negligible because the content of total flavin...
Fig. 3. Effect of caffeine and glucose on riboflavin production by non-growing cells of a non-flavinogenic strain of *E. ashbyii*. For experimental conditions see the footnotes of Fig. 1.

was far above those in mycelia.

Next, the effect of caffeine and glucose on the nucleotide pools in the non-flavinogenic strain was examined by using Dowex 1 x 2 (formate^-) column chromatography.

As shown in Fig. 4, NAD and AMP were clearly observed on the chromatograms, and the ratios of NAD to AMP were almost similar in the four experimental

Fig. 4. Effect of caffeine and glucose on nucleotide pools in non-growing cells of a non-flavinogenic strain of *E. ashbyii*. Acid soluble extract (10 ml) from the mycelia which were incubated under the conditions shown in Fig. 3 was applied to a column on Dowex 1 x 2 (formate^-). Column chromatography was carried out under the same systems as those in Fig. 2.
conditions indicated. However, G₃A did not accumulate at all, even in the glucose-free medium supplemented with 5 mM caffeine, despite of the fact that with the flavinogenic strain the compound accumulated markedly in the same medium. Accordingly, these findings suggest that the G₃A accumulated in the flavinogenic strain is not a degradation product from RNA, but a closely related compound to riboflavin formation.

(3) Detection of G₃A in the cells of E. coli, S. cerevisiae and N. crassa

E. coli, S. cerevisiae and N. crassa, which have a low flavinogenic activity, were cultivated in the basal medium for the definite hour and submitted to the same non-growing cell experiments with caffeine in the glucose-free medium as the case of the flavinogenic strain of E. ashbyii. The results are shown in Fig. 5.

A clear separation of NAD and AMP was obtained on the chromatograms. The amounts of NAD and AMP were almost identical in these cells of E. coli and S. cerevisiae. The facts suggest that the compositions of intracellular nucleotide pools are almost similar even in different species like the bacterium and the yeast. However, these microorganisms did not accumulate G₃A intracellularly as seen in this figure.

Fig. 5. Effect of caffeine and glucose on nucleotide pools in non-growing cells of E. coli and S. cerevisiae. Acid soluble extract (10 ml) from the microorganisms, which were incubated in the glucose-free medium supplemented with 5 mM caffeine, was respectively applied to a column on Dowex 1 × 2 (formate⁻). The column was developed under the same conditions as those in Fig. 2.
Moreover, the existence of G₃A was investigated also with *N. crassa* in the glucose-free medium containing caffeine in the same way as described above, but G₃A was not detected in the mycelia as in the non-flavinogenic *E. ashbyii*, *E. coli* and *S. cerevisiae*.

**DISCUSSIONS**

It has been known that caffeine inhibits the enzyme activity of cyclic-AMP phosphodiesterase [EC 3.1.4.17] (*15, 16*) or shows mutagenic action on DNA and RNA (*9, 10*) as its biological actions. However, not only cyclic-AMP but also caffeine derivatives such as caffeine nucleotides were not accumulated in non-growing mycelia of a flavinogenic *E. ashbyii* with the addition of caffeine. In the meanwhile, G₃A was accumulated in non-growing cells of the flavinogenic strain of *E. ashbyii*, especially in a glucose-free medium with caffeine. The structural features of G₃A suggested the possibility that the compound might be a degradation product of RNA. Therefore, in the present paper, the manner in which G₃A plays a role in riboflavin biosynthesis was investigated using a non-flavinogenic *E. ashbyii*, a bacterium, *E. coli*, a yeast, *S. cerevisiae* and a fungus, *N. crassa*, in addition to the flavinogenic strain of *E. ashbyii*.

In a non-flavinogenic strain of *E. ashbyii*, G₃A did not accumulate at all, in spite of the incubation of mycelia in a glucose-free medium supplemented with caffeine. Accordingly, the results suggest that G₃A is not a degradation product from RNA and DNA in *E. ashbyii*. If G₃A was a degradation product, it would be accumulated also in the non-flavinogenic strain of *E. ashbyii*, which is assumed to have the similar genes except for the regions related to riboflavin biosynthesis.

Furthermore, the accumulation of G₃A was also not observed in the cells of *E. coli*, *S. cerevisiae* and *N. crassa* which are well known microorganisms with a lower flavinogenic activity as well as the non-flavinogenic strain of *E. ashbyii*.

Accordingly, it is valid to consider the effect of caffeine used at a concentration of 5 mM as follows: the drug does not function as inducer of mutation so as to result in the accumulation of G₃A as a degradation product of DNA and/or RNA but becomes an inhibitor for a certain step in flavinogenesis so as to cause the accumulation of G₃A as a metabolite involved in the riboflavin biosynthetic pathway. The present authors found that guanosine triphosphate (GTP) is a direct nucleotide precursor in the biosynthetic pathway of riboflavin by experiments with labeled purines (*17, 18*). Thus, it is possible that G₃A is formed *in vivo* by the reaction of GTP with another substrate like adenosine triphosphate (ATP). Furthermore, it is of great interest that G₃A is a naturally occurring compound as seen in its accumulation in a glucose-free medium without caffeine (Fig. 2(2)), in which riboflavin formation proceeds at a lower level. This indicates that G₃A may be an accumulated compound closely related to riboflavin formation under a restricted condition.
In general, the mutation induced by caffeine appears to occur only at much higher concentrations (over 50 mM) compared to the concentration of 5 mM in this experiment (9, 10). There are no reports showing that only such a dinucleotide as GpA was specifically accumulated, even if mutation should be caused by the addition of caffeine over a concentration of 50 mM. In this sense, the results obtained in the present experiments appear to be specific.

Furthermore, the effects of caffeine on purine and nucleotide pools in a high flavinogenic E. ashbyii were examined by column chromatography on Dowex 50W × 4(H⁺) under various conditions used in the present experiments. However, there was no accumulation of any unknown compound on the chromatograms revealing almost same patterns in the addition of caffeine and glucose singly or in combination, although the accumulation of caffeine was found in the supplementation of caffeine.

Therefore, the results obtained suggest that GpA is a compound closely related to riboflavin formation, the accumulation of which is clearly observed in a flavinogenic strain of E. ashbyii under the inhibition of riboflavin formation. However, it remains obscure whether GpA takes part in a certain step of flavinogenesis. Further investigation is needed to elucidate this problem.

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