RELATION BETWEEN SUGAR METABOLISM AND RIBOFLAVIN FORMATION IN NON-GROWING CELLS OF *EREMOTHECIUM ASHYII*  

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Summary The present experiments were carried out to investigate the relation between sugar metabolism and riboflavin formation in non-growing cells of *Eremothecium ashbyii* incubated with various sugars (related compounds) and purines. Glycerol, gluconate and glucono-δ-lactone markedly stimulated riboflavin formation with increasing concentrations up to 0.5% or with increasing incubation times, but above the concentration range the effects of these substances on flavinogenesis were different. Ribose, xylose and ribitol brought about a weak stimulation of riboflavin formation in a concentration range of 0-0.2%. Glucose and fructose enhanced flavinogenesis in a concentration range of 0-0.5% but were inhibitory above the range. Glucosamine strongly restricted riboflavin formation in lower concentrations and the inhibition effect occurred immediately after its addition. The inhibition of riboflavin formation due to glucosamine (0.15%) was almost completely recovered by glucose (1.0%) but not by glycerol. Caffeine (5 mM) reduced the yields of riboflavin to a fairly greater extent. The decrease was reversed not by xanthine, guanine and theobromine but by glycerol, ribose and glucose, especially by glycerol (0.5%). Accordingly, caffeine was considered to inhibit a pentose phosphate pathway and glucosamine to inhibit a glycolytic pathway closely related to flavinogenesis.

BACHER and LINGENS (1, 2) and LOGVINENKO et al. (3) isolated two pyrimidines, 2,4,5-triamino-6-hydroxypyrimidine and 4-ribitylamino-2,5-diamino-6-hydroxypyrimidine, from riboflavin-deficient mutants of yeast. They suggested that...
these compounds are intermediates originating from a cleavage reaction at carbon 8 of the imidazole ring of the GTP molecule. The present authors showed that the direct purine precursor of riboflavin is guanosine triphosphate (GTP) (4-6), which is converted to an immediate intermediate of 6,7-dimethyl-8-ribityllumazine, 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine (7, 8). Accordingly, studies on the biosynthetic pathway of pyrimidine and pyrazine rings of riboflavin have greatly progressed.

In contrast, there are abundant reports along different lines that acetate (9, 10), acetoin (11), the members in a pentose phosphate cycle (12), the sugars in a glycolytic pathway (13) or the metabolites in a 2,3-butanediol pathway (14), have high incorporation effects into the o-xylene ring of riboflavin. While, it was reported recently that the ribose moiety of guanosine incorporates without dilution into the ribityl side chain of riboflavin (15). Furthermore, the possibility was found that the added ribitol reacts with endogenous purine or pyrimidine precursor to form the ribityl-intermediate in flavinogenesis (16-18). Accordingly, the origins of the 4-carbon unit and the ribityl side chain remain to be solved.

In the present paper, the relation of sugar metabolism to flavinogenesis and the role of caffeine in the sugar metabolism related to flavinogenesis were studied by using non-growing cells of Eremothecium ashbyii.

MATERIALS AND METHODS

Materials. Sugars, the related compounds and purines were purchased from Nakarai Chemicals, Ltd., Kyoto. Other chemicals and nutrients used for cultivation were of best quality available.

Organism and fermentation. Eremothecium ashbyii (IFO 0944) were obtained through the courtesy of Institute for Fermentation, Osaka and subcultured on an agar slant every week. A loopful of the mycelia from the agar slant was transferred to the preculture medium and cultured for 1 day. An appropriate volume of the culture was further transplanted to the basal medium, then cultured for 1 day (3). The mycelial pad obtained by filtration was submitted to the non-growing cell experiment.

Non-growing cell experiment. As previously reported (4, 19, 20), the mycelia obtained at a logarithmic phase were suspended in 0.1 M phosphate buffer (pH 6.8) and starved by mild shaking at 27°C for 8 hr. After starvation, the mycelia were suspended again in the same buffer containing test compounds at the ratio of 1 g wet mycelia/20 ml buffer, and vacuum infiltration of the test compounds into the mycelia was done. Thereafter, non-growing cell incubation was carried out with moderate shaking for the indicated times at 27°C.

Determination of riboflavin content formed. The flask containing the mycelial suspension was placed on a water bath and heated in the dark at 80°C for 10 min. A clear filtrate was obtained by filtrating the mixture. To determine
the content of flavins formed, the filtrate was diluted 8-fold with distilled water and the absorbance of the solution at 450 nm was measured. The total flavin content (sum of riboflavin, FMN and FAD) was spectrophotometrically calculated from the absorbance using 11.2 as a millimolar extinction coefficient of riboflavin at 450 nm.

RESULTS

1. Effects of sugars and related compounds at varied amounts on riboflavin formation

Non-growing cell incubation was carried out for 20 hr in the presence of various sugars and the related compounds at varied amounts. Results are given in Fig. 1. The addition of glycerol, gluconate and glucono-δ-lactone noticeably stimulated the formation of riboflavin with increasing concentrations up to 0.5%. Among these compounds, glycerol was most favourable for flavinogenesis up to

Fig. 1. Effects of sugars and related compounds at varied amounts on riboflavin formation in non-growing cells of E. ashbyii. The mycelia (1 g) of E. ashbyii starved by mild shaking for 8 hr in a phosphate buffer (pH 6.8), were suspended in the same buffer (20 ml) containing each test compound at the indicated concentration in a 200-ml Erlenmeyer flask, then incubated with moderate shaking for 20 hr at 27°C. Total flavin, after extraction on a water bath, was determined by reading the absorbance at 450 nm.

Fig. 2. Effects of sugars and related compounds on riboflavin formation in non-growing cells of E. ashbyii. The mycelia (20 g) of E. ashbyii starved by mild shaking for 8 hr in a phosphate buffer (pH 6.8), were suspended in the same buffer (400 ml) containing sugars (1%) in a 2-L Sakaguchi flask and were incubated with moderate shaking. Each ten ml of the cell suspension was harvested at the indicated incubation times. Total flavin, after extraction on a water bath, was determined by reading the absorbance at 450 nm.
2.0% but glucono-δ-lactone rather decreased the formation of riboflavin in concentrations higher than 0.5%. These compounds showed the same behavior in flavinogenesis in a 0–0.5% concentration range. Ribose, xylose, and ribitol in this order gradually enhanced the formation of riboflavin to a concentration of 2.0%, respectively. While, glucose and fructose stimulated the formation up to a 0.5% concentration, but above the level they reduced the formation of riboflavin. Glucosamine strongly inhibited the formation in low concentration. The inhibition degree was 85% in the addition of 0.25% glucosamine.

Accordingly, these sugars can be divided into four groups according to their stimulation effects on flavinogenesis: group 1, the highest stimulant—glycerol, gluconate, and glucono-δ-lactone; group 2, a high stimulant—ribose, xylose, and ribitol which show the less stimulation effect than the group 1; group 3, a normal stimulant—glucose and fructose; group 4, a very rigid inhibitor—glucosamine.

2. Effects of sugars and related compounds on riboflavin formation during non-growing cell incubation

Non-growing cell incubation was carried out for a definite incubation period in the presence of sugars and the related compounds at 1%. Riboflavin formation was followed by sampling a constant volume of the mycelial mixture and determining the riboflavin content. Results are given in Fig. 2. Glycerol remarkably stimulated riboflavin formation during incubation. Other sugars and the related compounds showed complicated stimulation effects with the incubation times. However, glycerol, gluconate, and glucono-δ-lactone, or glucose and ribose, indicated a similar attitude in flavinogenesis during incubation. The former compounds markedly enhanced the formation of riboflavin after 16 hr incubation, but the latter compounds retained a mild stimulation for the formation during incubation. While, glucosamine continued to rigidly inhibit the formation during the course of incubation. These results appear to give a validity for the classification of these additives into four groups in connection with flavinogenesis done in the previous section.

Next, the flavinogenic effects of glucose, gluconate, and glucosamine (1% respectively) added at 12 hr of incubation were examined in the presence or absence of glucose added initially. Results are given in Figs. 3 and 4. As shown in Fig. 3, glucose enhanced riboflavin formation by 110% above that of the control at 36 hr of incubation. Gluconate almost linearly stimulated the formation of riboflavin after the addition while glucosamine completely restricted riboflavin formation immediately after the addition. Whereas, as seen in Fig. 4, the addition of glucose at the start of incubation stimulated riboflavin formation by 60% compared to that without glucose at 12 hr of incubation. However, the further addition of glucose to the glucose-supplemented medium at 12 hr of incubation, when flavinogenesis logarithmically proceeds in the non-growing cells, rather inhibited the formation of riboflavin. In this case, gluconate indicated the same
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Fig. 3. Effects of glucose, gluconate and glucosamine on riboflavin formation in non-growing cells of *E. ashbyii*. The mycelia (20 g) of *E. ashbyii* starved by mild shaking for 8 hr in a phosphate buffer (pH 6.8), were suspended in the same buffer (400 ml) in a 2-1 Sakaguchi flask, then incubated with moderate shaking at 27°C for 12 hr. At 12 hr of incubation, the cell suspensions were divided into four portions (around 100 ml), which were respectively placed in a 500-ml Sakaguchi flask. Glucose, gluconate or glucosamine in solid forms was respectively added to each flask to give a final concentration of 1%. The mycelial mixture was incubated again for further 24 hr. Ten ml of the mixture were taken away at the indicated times during incubation. Total flavin, after extraction on a water bath, was determined by reading the absorbance at 450 nm.

Fig. 4. Effects of glucose, gluconate and glucosamine on riboflavin formation in non-growing cells of *E. ashbyii*. The mycelia (20 g) of *E. ashbyii* starved by mild shaking for 8 hr in a phosphate buffer (pH 6.8) were suspended in the same buffer (400 ml) containing 1% glucose in a 2-1 Sakaguchi flask. The subsequent experimental procedures were the same as those in Fig. 3.

trends as those of glucose after the addition. While, the addition of glucosamine promptly caused the inhibition of the formation in spite of the addition of glucose at the start of incubation. Moreover, as seen in Figs. 3 and 4, the addition of glucose at the start or at 12 hr incubation provided the same yields of riboflavin at 36 hr incubation, during which time flavinogenesis was in the beginning of the stationary phase. These results indicate that glucose is much more effectively utilized at the logarithmic stage of riboflavin biosynthesis.

3. Effects of sugars and related compounds on riboflavin formation inhibited by caffeine or glucosamine

Examination was made to determine whether or not the inhibition of ribo-
flavin formation by caffeine or glucosamine initially added was reversed by the addition of sugars and the related compounds at 12 hr incubation with non-growing cells of E. ashbyii.

As seen in Fig. 5, the addition of glycerol, glucose and ribose at 12 hr after incubation, stimulated the formation of riboflavin in this order. On the other hand, the addition of glucosamine (0.25%) at the start of incubation strongly inhibited the formation of riboflavin during incubation. The inhibition was restored to some extent by the addition of glycerol, glucose or ribose (1%) at 12 hr incubation. However, the restoration was effective in the order of glycerol, glucose and ribose, which was the same trend as in the stimulation of riboflavin formation.

While the addition of caffeine (5 mM) at the start of incubation reduced riboflavin formation by 45% at 12 hr incubation, the addition of the sugars at that time greatly recovered the inhibition by caffeine. However, the order of these compounds according to recovery effects differed from that in the stimulation effects: glycerol, glucose and ribose in this order stimulated the formation but glycerol, ribose and glucose in this order recovered the inhibition by caffeine. Especially, glucose showed no recovery effect. The results suggest that caffeine inhibits the pentose phosphate cycle closely related to flavinogenesis.
4. Effects of flavinogenic purines on riboflavin formation inhibited by caffeine

The present authors previously reported that the additions of xanthine, guanine or theobromine to the glucose-supplemented medium stimulate riboflavin formation both in growing cells and in non-growing cells of *E. ashbyii* (21). Effects of xanthine, guanine and theobromine on the inhibition of riboflavin formation by caffeine were examined with non-growing cells of *E. ashbyii*. In these experiments, no sugar was added to the medium during both starvation and non-growing cell incubation.

![Graph](image)

**Fig. 6.** Effects of xanthine, guanine or theobromine at varied amounts on riboflavin formation inhibited by caffeine (5 mM). The mycelia (1 g) of *E. ashbyii* starved by mild shaking for 8 hr in a phosphate buffer (pH 6.8), were suspended in the same buffer (20 ml) containing 5 mM caffeine and the indicated concentrations of xanthine, guanine or theobromine (without caffeine in the control) and was incubated with moderate shaking in a 200-ml Erlenmeyer flask at 27°C for 18 hr. Total flavin, after extraction on a water bath, was determined by reading the absorbance at 450 nm.

As seen in Fig. 6, the addition of xanthine showed no effect on flavinogenesis with increasing concentrations up to 10 mM. While, the addition of caffeine (5 mM) inhibited riboflavin formation by 50%. The inhibition was not restored at all by the supplementation of xanthine at varied amounts. The same results were obtained with guanine and theobromine, although the addition of the latter compound rather inhibited the formation of riboflavin with increased concentrations. These results indicate that purine exerted a stimulation effect on flavinogenesis only with the concomitant supplementation of sugar under the experimental conditions. Accordingly, it appears that the endogenous amounts of the purine nucleotides, which are known to be precursors of the isoalloxazine ring (1–3), are sufficient but those of the sugars as the sources of the 4-carbon unit and the ribityl side chain are deficient for the formation of riboflavin in the non-growing cells of *E. ashbyii*. 
5. Effects of glucose and glycerol at varied amounts on riboflavin formation inhibited by caffeine or glucosamine

The inhibition of flavinogenesis due to caffeine was not recovered by the addition of purines to the sugar-free medium. Thus, the effects of various concentrations of glucose and glycerol added on the inhibition of riboflavin formation by caffeine or glucosamine were studied with non-growing cells of *E. ashbyii*.

As shown in Figs. 7 and 8, riboflavin formation was inhibited 75\% by the ad-

![Fig. 7. Effects of glucose at varied amounts on riboflavin formation inhibited by caffeine (5 mM) or glucosamine (0.15\%). The mycelia (1 g) of *E. ashbyii* starved for 8 hr in a phosphate buffer (pH 6.8) were suspended in the same buffer (20 ml) containing 5 mM caffeine or 0.15\% glucosamine and the indicated concentrations of glucose (without caffeine and glucosamine in the control). The suspension was incubated in a 200-ml Erlenmeyer flask for 18 hr at 27°C. After extraction on a water bath, total flavin was determined by reading the absorbance at 450 nm.](image)

![Fig. 8. Effects of glycerol at varied amounts on riboflavin formation inhibited by caffeine (5 mM) or glucosamine (0.15\%). The experimental conditions were the same as in Fig. 7, except that glycerol was used in place of glucose.](image)
dition of caffeines (5 mM) and 87% by glucosamine (0.15%) compared to the control. The addition of glucose considerably reversed the inhibition by caffeine but gradually restored the inhibition by glucosamine with its increasing concentrations. The inhibition by caffeine was recovered by 90% at a 0.3% concentration of glucose and that by glucosamine was completely recovered at a 2.0% concentration of glucose compared to the control without glucose. On the other hand, the inhibition due to caffeine was effectively restored with increased concentrations of glycerol showing almost the same yields of riboflavin as those in the control, at a 0.5% concentration of glycerol. However, inhibition due to glucosamine was not recovered at all with increasing concentrations of glycerol to 2.0%. The effective recovery of the caffeine inhibition by glucose and glycerol, especially glycerol, may indicate that caffeine inhibits any step of a pentose phosphate pathway related to flavinogenesis. While, the almost complete recovery of the glucosamine inhibition by glucose but not glycerol may demonstrate that glucosamine inhibits any step of a glycolytic pathway in connection with flavinogenesis.

DISCUSSION

The roles of sugars and the related compounds as the building blocks of riboflavin molecule have been studied by many researchers (9-18). But, the origins of the 4-carbon fragment and the ribityl side chain are still unclear. This must indicate the complex profiles of sugar metabolism in relation to flavinogenesis. In the present paper, an attempt was done to obtain the information about the origins of the 4-carbon unit and the ribityl group using non-growing cells of Eremothecium ashbyii in the presence of various sugars and the related compounds.

Experiments in the supplementation of sugars and the related compounds at varied amounts enabled us to classify these compounds into four groups based on their flavinogenic activities: the metabolites in an oxidative pathway of the pentose phosphate cycle (glycerol, gluconate and glucono-δ-lactone) belonging to group 1; the metabolites in a non-oxidative pathway of the pentose phosphate cycle (ribose, xylose and ribitol) to group 2; the metabolites in a glycolytic pathway (glucose and fructose) to group 3; a potent inhibitor (glucosamine) to group 4.

Furthermore, experiments following the flavinogenic effects of sugars and the related compounds at 1% during incubation demonstrated that at an early stage of flavinogenesis the metabolite in group 3 is more effective than the metabolites in group 1, but at the later stage in which riboflavin formation proceeds logarithmically, the latter members contribute much more effectively to the biosynthesis of riboflavin than the former member. Accordingly, it may be possible to consider that a glycolytic pathway mainly functions as the sources of the 4-carbon unit and the ribityl side chain of riboflavin for the gradual formation of riboflavin, but the pentose phosphate cycle, especially the oxidative pathway, plays a significant
roles in the sugar metabolism supplying two building blocks for the active formation of riboflavin. In sugar metabolism, glucose, being the sugar common to both the pentose phosphate pathway and the glycolytic pathway, appears to stimulate riboflavin formation by the gradual shift with flavinogenesis from the glycolytic pathway to the pentose phosphate pathway.

Although the compounds in group 2 can become members in the pentose phosphate cycle as well as the compounds in group 1, the former compounds did not exhibit such a high flavinogenic activity as the latter compounds. This may indicate that endogenous pentose phosphates are much more effectively provided by an oxidative pathway of the pentose phosphate pathway (22) than by a salvage pathway (23–25) of endogenous ribose, xylose and ribitol. Otherwise, the results may demonstrate the existence of the conversion pathway from 6-phospho-D-gluconate to pyruvate and 3-phosphoglycerate in the mold as found in Pseudomonas fluorescens (26).

On the other hand, it is to be noted that glucosamine rigidly inhibited riboflavin formation in a lower concentration for the prolonged incubation period. Furthermore, glucosamine completely restricted the formation of riboflavin simultaneously with the addition (1%) at 12 hr after incubation. The inhibition was not affected when glucose (1%) was added at the start of incubation. Moreover, the inhibition of riboflavin formation which was caused by the addition of glucosamine (0.25%) at the beginning of incubation was reversed to only a small extent by the addition of glycerol, glucose and ribose (1%, respectively) at 12 hr incubation, whereas the inhibition was reversed with increasing concentrations of glucose but not glycerol initially added, showing the complete recovery by the addition of more than 1% glucose. Thus, glucosamine appears to become a potent inhibitor for the glucose metabolism involved in the glycolytic pathway in connection with flavinogenesis. However, the results obtained do not seem to be able to explain at present by the known action mechanism of glucosamine for sugar metabolism (27–29) and for cell growth (30).

The present authors previously reported that the trimethyl xanthine, caffeine, inhibited riboflavin formation by a high flavinogenic mold, Eremothecium ashbyii (21) and simultaneously caused the accumulation of a novel dinucleotide, guanine ribonucleotidyl-(3'-5')-adenosine (GpA) (31), in the mycelia. In the present paper, the relation between caffeine and the sugar metabolism related to flavinogenesis was studied. The addition of 5 mM caffeine at the start of incubation showed a 48.3% inhibition of riboflavin formation at 12 hr incubation. The addition of glucose, glycerol and ribose (1%, respectively) at 12 hr effectively reversed the inhibition by caffeine added originally. The better recovery by glycerol and ribose at the logarithmic phase of flavinogenesis especially, suggests that the inhibition of riboflavin formation by caffeine occurs in a pentose phosphate pathway. The involvement of the caffeine inhibition in the sugar metabolism related to flavinogenesis is supported also by the facts that the caffeine inhibition
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is completely reversed by the addition of glucose and glycerol, especially glycerol, but not by the addition of flavinogenic purines, xanthine, guanine and theobromine, in increasing concentrations. Accordingly, it is considered that added caffeine blocks the sugar metabolism being the origins of the o-xylene ring and the ribityl group of riboflavin, leading the inhibition of riboflavin formation, so that GpA is formed as the accumulated form of excess nucleotide precursor, GTP.

Glycerol, which can enter the pentose phosphate cycle and the glycolytic pathway, was much more effective than glucose being also a common sugar to both pathways and than ribose being a possible direct precursor of the ribityl side chain of riboflavin. However, glycerol could not recover the inhibition of riboflavin formation by glucosamine (0.15%) at all even by adding it in high concentration (2%). Accordingly, the high stimulative effect of glycerol, the rigid inhibitory effect of glucosamine on flavinogenesis and the inhibition mechanism of caffeine in the sugar metabolism closely related to flavinogenesis may provide a clue to elucidate the origins of the 4-carbon fragment and the ribityl side chain required for riboflavin formation.

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