Note

Effects of Riboflavin on 6,7-Dimethyl-8-ribityllumazine Formation in Growing Cells of a Riboflavin-Adenine-Deficient Mutant, *Bacillus subtilis*

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Summary The effects of riboflavin on the formation of 6,7-dimethyl-8-ribityllumazine (DMRL) were examined using the growing cells of a riboflavin, adenine deficient-mutant, *Bacillus subtilis*. The administration of low concentrations (50–500 ng/mL) of riboflavin to the basal medium significantly inhibited the formation of DMRL without any change in growth. Especially, the supplementation of more than 250 ng/mL riboflavin completely restricted the formation of DMRL under the experimental conditions. The results clearly indicate that a rigid negative feedback inhibition is operative for the biosynthetic pathway of riboflavin in *B. subtilis*.

Key Words 6,7-dimethyl-8-ribityllumazine, riboflavin, *Bacillus subtilis*, feedback inhibition

The biosynthetic mechanisms of riboflavin formation have been investigated using many microorganisms and various experimental methods for the past half century (1–3). The precursor and several pyrimidine intermediates on the biosynthetic pathway were isolated and identified in bacteria and fungi before 1980. Since then, the detailed reaction mechanisms between the primary intermediates have been further investigated (3).

The regulation of riboflavin formation has been reported in inhibition experiments related to iron with whole cells (4, 5) and crude enzymes (6, 7) from *Pichia guilliermondii*, and it is possible that it can be performed at any reaction step on riboflavin biosynthetic pathway by flavin mononucleotide (FMN) and/or flavin adenine dinucleotide (FAD) in *B. subtilis* (8, 9) and *P. guilliermondii* (10). Moreover, regulation was also reported at the levels of transcription and translation in the biosynthesis of riboflavin in bacteria (11) and fungus (12). Therefore, here I attempted to confirm the possibility of a negative feedback inhibition of riboflavin formation using a riboflavin-adenine double less-mutant of *B. subtilis*.

The reagents for fermenting of the microorganisms were purchased from Wako Pure Chemical Industries, Co., Ltd., Japan. The other chemicals used were of the best quality available. A riboflavin-adenine-deficient mutant, *Bacillus subtilis* AJ1988, was the gift of Ajinomoto Co., Ltd., Japan. The media and cultivation of *B. subtilis* were the same as in the previous paper (13).

The growth of the bacteria was expressed by the absorbance of a 31-fold diluted culture broth at 550 nm and specified times. After centrifugation, the amounts

of formed DMRL were calculated by the absorbance of the 31-fold diluted supernatant $\times 31 \times 1/10.2$ (molar extinction coefficient of DMRL) at 408 nm, and expressed in terms of mM. The pH values of the medium were measured with a Horiba pH meter (Mode F-23).

The riboflavin-adenine double-less mutant of *B. subtilis* proved to be lacking the riboflavin synthase reaction and to produce large amounts of the precursor of riboflavin, DMRL. This indicates the strong possibility that the inhibition of DMRL formation by the end product, riboflavin and its derivatives as such, is a negative feedback inhibition in the bacteria. Thus, the mutant cells were used in the present experiments.

The effects of the administration of various concentrations (0–500 ng/mL) of riboflavin to the basal medium on the growth, formation of DMRL and medium pH were examined 3 d after cultivation. The results are shown in Fig. 1. The growth reached a maximum at around 50 ng/mL, and thereafter remained almost unchanged to the concentration of 500 ng/mL. The formation of DMRL reached a maximum at 5 ng/mL and thereafter rapidly decreased to the concentration of 50 ng/mL, then the concentration was gradually reduced to 250 ng/mL, at which time the formation of DMRL ceased completely. Over the lower concentration range of riboflavin, the maximal formation (0.51 mM) of DMRL was detected at 5 ng/mL (0.013 mM) riboflavin, and thereafter reduced to the amount of 0.22 mM at 50 ng/mL (0.13 mM) riboflavin, which was sufficient for maximum growth. Thus, the bacteria appear to produce large amounts of DMRL. However, rigid negative feedback inhibition proved to function at the earlier stages of growth in the bacteria.

In contrast, the effects of 50 or 300 ng/mL riboflavin

Fig. 1. Fluctuation of growth, riboflavin formation and medium pH with increasing concentrations of riboflavin. These parameters were determined on a basal medium with riboflavin ranging from 0 to 500 ng/mL 3 d after cultivation. For experimental conditions see details in the text.

Fig. 2. Fluctuation of growth, riboflavin formation and medium pH with increasing culture times in presence of 50 or 300 ng/mL riboflavin. These parameters were determined on the basal medium containing 50 or 300 ng/mL riboflavin during the cultivation period for 3 d. For experimental conditions see details in the text.

on the growth, DMRL formation and medium pH were examined during the cultivation period for 3 d. The results are given in Fig. 2. The addition of 50 and 300 ng/mL riboflavin afforded almost no difference in growth and medium pH, but both significantly changed the behavior of DMRL formation during the cultivation period for 3 d, showing about 50% and 100% inhibition on the 3rd day as compared to the amounts of DMRL at the start of the experiments, respectively.

Therefore, the administration of more than 5 ng/mL riboflavin began to restrict the formation of DMRL, and that of more than 50 ng/mL riboflavin afforded almost no effect on growth but remarkably inhibited only DMRL formation. Moreover, the addition of more than 250 ng/mL riboflavin completely restricted only DMRL formation without any change in growth.

Accordingly, the present experiments with the grow-

ing cells of *B. subtilis* provide a clear-cut result that the rigid negative feedback inhibition by riboflavin and/or its derivatives (FMN and FAD) is also operative for the riboflavin biosynthetic pathway in *B. subtilis*.

The elucidation of regulation mechanisms of riboflavin formation in *B. subtilis* is being investigated.

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