THE RELATION BETWEEN PURINE METABOLISM AND FLAVINOGENESIS IN *EREMOTHECIUM ASHBYII*

THE IDENTIFICATION OF S-ADENOSYLMETHIONINE AND S-ADENOSYLMHOCYSTEINE ACCUMULATED IN NON-GROWING CELLS OF *E. ASHBYII*

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Summary When adenine was added to the non-growing cell medium of *Eremothecium ashbyii*, riboflavin production of the mold was increasingly inhibited with increasing concentration of adenine. Under these conditions, a cationic compound was accumulated in the mycelia. The compound was isolated from the mycelia and highly purified. The purified compound was proved to be S-adenosylhomocysteine through IR analysis. In the control experiment, or in the addition of other purines which stimulated riboflavin production of the mold, another cationic compound was accumulated in the non-growing cells. The compound was largely accumulated in the presence of both adenine and methionine, isolated from the mycelia and purified. The purified compound was concluded to be S-adenosylmethionine through IR and NMR analyses. The significance of these compounds on the riboflavin biosynthetic pathway was argued under "DISCUSSION."

As reported previously, adenine inhibited riboflavin production while other purines stimulated riboflavin production in non-growing cells of *Eremothecium ashbyii*. Under these conditions, two unknown compounds were accumulated in the mycelia; one, referred to as compound X, is the compound accumulated upon the adenine addition and the other, referred to as compound Y, is the compound accumulated upon the addition of guanine, xanthine or hypoxanthine or also where no addition was made. Furthermore, the latter compound was markedly accumulated by the addition of methionine and adenine in combination. These compounds were isolated and identified by means of paper chromatography and ultraviolet light absorption spectra as indicated in our previous paper (1). The

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results revealed that compound X was S-adenosylhomocysteine and compound Y was S-adenosylmethionine.

In the present experiment, these compounds were isolated again and purified to a great extent from mycelia of *E. ashbyii*. The structures of the purified compounds were clarified through IR and NMR analyses.

MATERIALS AND METHODS

Materials. S-Adenosylhomocysteine and S-adenosylmethionine were purchased from the Sigma Chemical Company. All other chemicals used in this experiment were of highest quality available. *Eremothecium ashbyii* guillermundii (IFO 0944) was obtained from the Institute for Fermentation in Osaka.

Methods. *Eremothecium ashbyii* was grown for 24 hr on a natural nutrient broth (pH 5.5) containing 2% glucose, 1% peptone, 0.3% yeast extract, 1.8% Tween 80, 0.2% KH_2PO_4 , 0.1% NaCl and 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The cells were harvested by filtration and washed several times with distilled water. The mycelial pad thus obtained was suspended in 0.1 M phosphate buffer, pH 6.8, containing 1% glucose at a ratio of 1 g wet weight per 20 ml buffer. Then, the mycelia were starved for 8 hr at 28°C with mild shaking. After starvation, the mycelia were harvested and washed again, and resuspended in the same buffer to which the test compounds described under "RESULTS" were added. After vacuum infiltration of the test compounds into mycelia, non-growing cell incubation was carried out for 12 hr at 28°C with moderate shaking (2). The accumulated compound X and compound Y were extracted, isolated and purified from the mycelia according to the procedures described under "RESULTS," based on those of DUERRE (3) and SCHLENK (4). IR spectra were recorded from a KBr pellet with a Hitachi Grating Infrared Spectrophotometer (model EPI-G3). NMR spectra were measured in D_2O containing DSS as an internal standard with a Hitachi High Resolution NMR Spectrometer (R-22).

RESULTS

Isolation and purification of compound X

In Fig. 1, the amounts of the accumulated compound X are plotted against the concentration of added adenine. The amounts of compound X increased with the increasing concentrations of adenine showing the maximum accumulation attained by the addition at above 5 mM concentration. Thus, adenine was added to the non-growing cell medium at a concentration of 5 mM for the isolation of compound X.

1. *Extraction.* Under the above conditions the mycelia were harvested through a filter paper after the 12 hr incubation, washed with distilled water and ground in a mortar for 10 min at 0°C with 2 volumes of distilled water and 0.4 volumes of 3 M HClO_4 . The clear supernatant was obtained by centrifugation at

Fig. 1. Accumulation of compound X at varied concentrations of added adenine. The mycelia incubated with the indicated concentrations of adenine were extracted with perchloric acid. The supernatant obtained by centrifugation was neutralized with potassium hydroxide as described in "RESULTS." The extract (10 ml) was applied to a Dowex 50W $\times$ 4 (H^+ , 200–400 mesh) column (1 $\times$ 8 cm). After washing with 120 ml of distilled water, 200 ml of 1 N HCl and then 300 ml of 2.5 N HCl were added to the column in stepwise elution systems with a mixing chamber containing 200 ml of distilled water. The transmittance of eluate at 253.7 nm was recorded by LKB-Uvicord (Type 4913 B) and the amounts of compound X were estimated from the absorbances and volumes of eluate.

8,000 rpm for 20 min, adjusted to pH 7.0 with 3 M KOH. The resultant precipitate, $KClO_4$, was removed by decantation and the extract was submitted to the following experiments.

2. *Alkali treatment.* Figure 2-a shows the elution pattern of compound X and compound Y on Dowex 50W $\times$ 4. The results indicate that the compound X fraction may contain a little amount of compound Y because both fractions were very near on the column chromatogram of Dowex 50W $\times$ 4. To avoid the contamination of compound Y, the extract was adjusted to pH 12 with 3 M KOH, and kept for 36 hr at 4°C. The solution thus treated was applied to the same column by the same method. The results were given in Fig. 2-b, which indicates that compound Y was completely decomposed and compound X fraction gave a single peak on the chromatogram.

3. *Column chromatography on Dowex 50W $\times$ 4 (H^+).* The alkali treated extract (880 ml) was applied to a Dowex 50W $\times$ 4 (H^+) column (3 $\times$ 48 cm), which was developed by the stepwise elution with 1.5 liter of 1 N hydrochloric acid and then the same volume of 3 N hydrochloric acid. Compound X appeared in the 3 N hydrochloric acid effluent following the elution of adenine. For precaution against the oxidation of compound X, each solvent contained 0.25 ml of β -thioglycol per liter.

4. *Phosphotungstic acid treatment.* Compound X was precipitated from the 3 N hydrochloric acid eluate by the addition of enough volume of 20% phos-

Fig. 2. Effect of alkali on degradation of compound Y. In Fig. 2-a, the perchloric acid extract from the mycelia supplemented with adenine was directly applied to a Dowex 50W $\times$ 4 column and in Fig. 2-b, the extract after alkali treatment was applied to the same column. The method of the column chromatography was the same as that described in Fig. 1.

phosphotungstic acid for complete precipitation. The precipitate was allowed to settle overnight at 4°C. The supernatant fluid was removed by decantation and centrifugation at 6,000 rpm for 10 min. The precipitate was then washed several times with cold distilled water.

5. *Removal of phosphotungstic acid.* After washing, the phosphotungstate of compound X was dissolved in an acetone-water mixture (1:1) to give a clear solution. The solution was treated with 4 volumes of *iso*-amylalcohol-ether (1:1) to remove phosphotungstic acid. The water layer was repeatedly treated with 2 volumes of the organic solvent. The organic solvent in the water layer was removed by extraction with several volumes of ether five times, and the remaining ether in the water layer was removed by N_2 gas flow. The aqueous solution thus obtained was centrifuged at 6,000 rpm for 10 min.

6. *Column chromatography on Sephadex G-10.* The supernatant obtained by centrifugation was applied to a Sephadex G-10 column (1.5 $\times$ 50 cm) which was developed with distilled water. The compound X fraction obtained was lyophilized. The dried matter was crystallized from distilled water, and the crystals were obtained by filtration, followed by washing with ethanol and ether. The purified crystals were submitted to IR-analysis. Through these purification steps, about 8 mg of

compound X was obtained from 400 g wet weight of mycelia.

Isolation and purification of compound Y

As described previously (1), compound Y was accumulated markedly by the addition of 10 mM methionine and 1 mM adenine in combination. Accordingly, the isolation and purification of compound Y was carried out from the mycelia under the conditions.

1. *Extraction.* Compound Y was extracted with perchloric acid by the same method as that used for compound X. The extract was provided for the following purifications without neutralization with potassium hydroxide.

2. *Ammonium reineckate treatment.* Ammonium reineckate solution, 2.5 volumes of 2%, was added to the perchloric acid extract. Compound Y was precipitated as reineckate salt, while compound X was not treated. The resulting precipitate was collected by centrifugation at 8,000 rpm for 10 min after decantation. The pellet was washed several times with cold distilled water with centrifugation and dissolved in 20 volumes of a mixture of acetone-1 N H₂SO₄ (1:1).

3. *Column chromatography on Dowex 50W×4 (H⁺).* The solution treated above was applied to a Dowex 50W×4 (H⁺, 100–200 mesh) column (2.2×23 cm). Compound Y was eluted with 3 N H₂SO₄ after washing with 1 N H₂SO₄, followed by 6 N H₂SO₄.

4. *Phosphotungstic acid treatment.* The compound Y fraction was treated with 20% phosphotungstic acid in the same way as compound X.

5. *Tetraethylammonium iodide treatment.* The resulting precipitate (phosphotungstate of compound Y), after washing with cold distilled water, ethanol and ether, was dissolved in 90% acetone. For removal of phosphotungstic acid from compound Y, 1 M tetraethylammonium iodide solution was added until the precipitation of phosphotungstate was completed. A supernatant was obtained by centrifugation, and adjusted to pH 5.8 with 1 M ammonia water; lyophilization followed. The yellowish oily compound was mixed with methanol to give a white granular material. The added methanol was distilled off *in vacuo* and methanol was again added. After distilling, the white granular material was washed with ethanol and ether. The material obtained was compound Y iodide, which was submitted to IR and NMR analyses. Finally, about 45 mg of compound Y iodide was prepared from 120 g (wet weight) of mycelia. All procedures were undertaken in the cold (0–4°C).

IR-spectrum of compound X

The IR spectrum of compound X shows specific absorptions attributable to the carboxyl group (1590 cm⁻¹), the amino groups (1630 cm⁻¹) and the hydroxy groups of sugar (1000–1150 cm⁻¹, broad) as seen in Fig. 3. The spectrum of authentic S-adenosylhomocysteine coincided well with that of the isolated compound X. In a previous report (1), it was verified that compound X contained

adenine, ribose and homocysteine as its components, and that R_f values of compound X were the same as those of authentic S-adenosylhomocysteine on the paper chromatograms. Accordingly, compound X is concluded to be S-adenosylhomocysteine.

Fig. 3. IR spectra of isolated compound X and authentic S-adenosylhomocysteine from KBr pellets.

Fig. 4. IR spectra of isolated compound Y iodide and authentic S-adenosylmethionine iodide from KBr pellets.

IR and NMR spectra of compound Y

Figure 4 shows the IR spectrum of compound Y, which is quite similar to that of compound X (S-adenosylhomocysteine). The differences in absorption between these compounds were in the facts that compound Y gave an absorption at 1770 cm^{-1} , and that the intensities of absorptions of these compounds at 1590 cm^{-1} and 1630 cm^{-1} showed an inverse relation. The characteristics of compound Y were also observed on the spectrum of authentic S-adenosylmethionine iodide.

These spectra showed a good agreement over the indicated wavelength. The difference in structures between S-adenosylhomocysteine and S-adenosylmethionine is in the fact that S-adenosylmethionine has a S-methyl group in its molecule. Therefore, the NMR spectrum of compound Y was recorded to detect the signal of the methyl group. The results are given in Fig. 5, and indicate that the isolated compound Y and authentic S-adenosylmethionine have a characteristic singlet

Fig. 5. NMR spectra of isolated compound Y iodide and authentic S-adenosylmethionine iodide in D_2O .

attributable to the S-methyl group ($\delta=3.1$). Furthermore, both spectra show signals of the anomeric proton of ribose ($\delta=6.2$) and the protons attached to the 2- and 8-carbon of adenine ($\delta=8.6$). Therefore, compound Y was identified as S-adenosylmethionine from these results and the results obtained in the previous report (1) indicating that compound Y contained adenine, ribose and methionine, and R_f values of compound Y, coincided well with those of authentic S-adenosylmethionine on the paper chromatograms.

The structures of isolated S-adenosylhomocysteine and S-adenosylmethionine were shown in Fig. 6.

DISCUSSION

Accumulation of S-adenosylmethionine in many microorganisms such as yeast and mold has been reported (5,6). Accordingly, it is reasonable that *Ere-*

Fig. 6. Structures of isolated S-adenosylhomocysteine and S-adenosylmethionine.

mothecium ashbyii, being a kind of mold, accumulates S-adenosylmethionine in cells, although, there are only a few reports on the accumulation of S-adenosylhomocysteine in molds. However, the increase of the accumulation of S-adenosylhomocysteine was observed with the increasing concentrations of added adenine in non-growing cells of *E. ashbyii*. Furthermore, the results obtained indicated that the inhibition of riboflavin formation was initiated simultaneously with the accumulation of S-adenosylhomocysteine due to the addition of adenine. On the other hand, S-adenosylmethionine was a naturally occurring compound detected in normal non-growing cells of *E. ashbyii*, and was consumed as riboflavin formation proceeded in these experiments. In the present report, 10 mM methionine, and 1 mM adenine were added together for accumulation and isolation of S-adenosylmethionine. Under these conditions, a large amount of S-adenosylmethionine was accumulated in cells, in which riboflavin formation was restored to a normal level, while S-adenosylhomocysteine was scarcely accumulated in spite of the presence of adenine with methionine. Thus, S-adenosylmethionine is considered to be a stimulative compound and S-adenosylhomocysteine to be an inhibitory compound of riboflavin formation.

Three different functions have been known as to the metabolism of S-adenosylmethionine; 1. a methyl group donor in transmethylation reactions (7,8). 2. a polyamine donor for spermidine synthesis (9,10). 3. the substrate in the cleavage reaction to 5'-methylthioadenosine and 4-carbon compound (11,12). The obtained results in the present experiments provide the possibilities of 1 and 3 in relation to the flavinogenesis. S-adenosylmethionine is well known as a methyl group donor in many transmethylation reactions, in which S-adenosylhomocysteine acts as a potent inhibitor. These facts suggest that transmethylation may be related to the riboflavin biosynthesis and S-adenosylhomocysteine may inhibit riboflavin formation as a result of inhibition of the transmethylation reaction.

On the other hand, a mechanism of S-adenosylmethionine has been reported in which the methionine moiety of S-adenosylmethionine is split off to give a 4-carbon fragment and 5'-methylthioadenosine in cells. In the mold, the carbon

sources are obligatory for the formation of riboflavin as 4-carbon, ribitol and energy sources. Accordingly, it will be worthwhile to study whether S-adenosyl-methionine takes a part in the transmethylation reaction related to the flavinogenesis or acts as the carbon source described above. We are conducting experiments on the basis of these considerations.

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