Inhibitor of Transfer Ribonucleic Acid Methy lases in 
the Differentiating Slime Mold  
Dictyostelium discoideum  

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Eight hours after the onset of morphogenesis, an inhibitor of transfer ribonucleic acid methylases appears in differentiating Dictyostelium discoideum. The inhibitor is also present in spores. Fifty per cent of the inhibiting activity is lost upon heating at 100 C for 5 min; it is nondialyzable and sensitive to trypsin.  

Alterations in transfer ribonucleic acid (tRNA) methylases have been reported in a variety of biological systems undergoing shifts in regulatory processes. Changes have been observed in insects during metamorphosis (2), in lens tissue during differentiation (S. J. Kerr and Z. Dische, Ass. Ophthalmol. Meeting, Tampa, Fla., 1968), on phage induction and infection (4, 16), in embryonic and neonatal tissue (8), in a variety of tumor tissues (5), and more recently in slime mold during morphogenesis (10). The altered patterns of methylation observed in vitro have been confirmed in vivo in a number of these systems (3, 15). However, the control mechanisms governing these changes are obscure.  

The colonizing slime mold offers a unique advantage for the study of a possible role of altered tRNA in regulatory processes. The organisms can be induced into synchronous morphogenesis and are ready sources for both the enzymes of tRNA synthesis and the tRNA itself. In previous studies we have observed both qualitative and quantitative changes in the tRNA methylases during the life cycle of Dictyostelium discoideum (10). After 8 hr of synchronous aggregation, extracts of the organism had a marked decrease in the capacity to methylate a heterologous tRNA substrate compared with extracts of the free-living amoeba.  

We now report that 8 hr after the onset of morphogenesis, an inhibitor of the tRNA methylases appears within the differentiating organisms.  

MATERIALS AND METHODS  
Organisms. D. discoideum Raper (American Type Culture Collection) was grown on petri dishes in association with Aerobacter aerogenes by the method of Sussman (13).  

Growth conditions. The growth medium contained  

- 2% agar,  
- 1% peptone (Difco),  
- 0.1% yeast extract,  
- 0.15% K$_2$HPO$_4$,  
- 0.1% MgSO$_4$, and  
- 1% glucose.  

Samples (1 ml) of slime mold spores (2 X 10$^6$ to 5 X 10$^6$) in 6- to 8-hr bacterial broth culture were distributed over the agar surface. After incubation at 22 C for 38 to 48 hr, the amoebae reached stationary phase. The amoebae were collected prior to aggregation in 10 ml of cold water per petri plate and were washed three times by differential centrifugation (5 min at 600 x g). This procedure eliminates most of the residual bacteria. The washed amoebae were suspended in water (4 X 10$^8$ to 6 X 10$^9$ per ml); 0.5-ml samples were spread on membrane filters (Millipore Corp., Bedford, Mass., type HAWP04700) resting on absorbent pads in 60-mm plastic petri dishes (13). The pads had been saturated with 2 ml of salt solution (0.05% MgCl$_2$, 0.15% KCl, 0.05% streptomycin sulfate). The petri dishes were placed in an incubator kept at 22 C.  

Preparation of cell-free extracts. Cells were collected after various stages of morphogenetic development, washed as above three or four times with ice-cold water, and disrupted in a 10-ke Raytheon apparatus (10 min). The spore mass was disrupted by grinding with alumina. A cell-free supernatant fraction, prepared by centrifuging the extracts at 100,000 x g for 1 hr, was used as the source of methylating enzymes.  

Enzyme assay. The methylase activity was assayed by measuring the incorporation of 14C methyl groups of S-adenosylmethionine into Escherichia coli B tRNA. The reaction mixture contained: tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, p$\text{H}$ 8.2 (100 $\mu$moles), MgCl$_2$ (10 $\mu$moles), mercaptoethanol (10 $\mu$moles), E. coli B tRNA (100 $\mu$g, General Biochemicals Corp., Chagrin Falls, Ohio), S-adenosyl-L-methionine (14C-methyl, 0.2 $\mu$c, specific activity 48 c/mole, International Chemical and Nuclear Corp.), and enzyme in a total volume of 1 ml. The control received no tRNA. After incubation at 37 C for 30 min, the reaction was terminated by the addition of 0.5 ml of ice-cold 10% trichloracetic acid. The tubes were chilled in ice, and after 20 to 30 min the
RESULTS

Methylase activity of cells aggregating on filters for 8 hr. Our earlier studies revealed differences in the characteristics of the enzyme extracts from amoebae and organisms which had been aggregating for 8 hr. At optimal protein concentrations, the capacity to introduce methyl groups was 65% higher in extracts from the amoebae than in similar extracts from the 8-hr aggregates. Moreover, with extracts of the latter, when optimal protein concentrations were exceeded, a diminution in methylase capacity was observed. This finding implies the presence in those extracts of some inhibitor(s) whose effect depends on its absolute concentration. A separation of inhibitor from mammalian tissues was readily effected by a fractional precipitation of the enzymes at pH 5 (S. J. Kerr, Fed. Proc., p. 866, 1969). The same procedure was also found to be effective with enzyme extracts of the slime mold.

The 100,000 × g supernatant fraction of extracts of the organisms aggregating for 8 hr was adjusted to pH 5.0 with 0.1 N acetic acid, and the precipitate was immediately centrifuged (10,000 × g for 10 min). The saturation characteristics of the enzyme in the precipitate was determined by dissolving it in 0.05 M Tris-hydrochloride, pH 8.2, and using the solution as a source of enzyme. At saturation level, the precipitate had a 50% higher methylase capacity than the original supernatant fraction. The addition of the pH 5.0 supernatant fraction (adjusted to pH 8.2, which had no detectable tRNA methylase activity) to the redissolved pH 5 precipitate resulted in the inhibition of the latter’s enzyme activity. These findings demonstrate the presence of some inhibitor of tRNA methylase in extracts of 8-hr aggregated amoebae (Fig. 1C).

Absence of the inhibitor from extracts of free amoebae and of cells aggregating on filters for 4 hr. The enzyme activity in extracts of 4-hr aggregates was not completely precipitated at pH 5.0. The supernatant fraction retained 20 to 30% of the methylase activity. When the precipitated enzyme was redissolved and assayed at saturation level, i.e., further addition of extract produced no increment in methyl group incorporation, its methylase capacity was found to be no different from the enzymes in the original 100,000 × g supernatant fraction (Fig. 1B).

An examination of extracts from free amoebae also failed to reveal the presence of any inhibitors of the tRNA methylases.

Presence of the inhibitor in spores. The terminal stages of fruiting were completed on filters in 24 hr. The harvest yielded a heterogeneous mixture consisting of spore mass together with the stalks. On precipitation at pH 5 of the 100,000 × g supernatant fraction of spore extracts, there was a 2.5-fold increase in the methylase capacity of the precipitate (Fig. 1D); the supernatant fraction was devoid of enzyme activity. The addition of the supernatant fraction after precipitation at pH 5 to the redissolved precipitate produced an inhibition of the tRNA methylase activity approximating that of the inhibitor from the 8-hr aggregates.

Nature of the inhibitor. The inhibitory material from the pH 5.0 supernatant fraction of 8-hr aggregated material was used for more detailed studies since it yielded the most reproducible preparations. That the inhibition of the tRNA methylases is proportional to the amount of pH 5 supernatant fluid added is evidenced by the data presented in Fig. 2.

Fifty per cent of the activity was lost upon heating at 100 C for 5 min. The inhibitor was nondialyzable and was stable during storage at −15 C. Exposure of the inhibitor to trypsin for 30 min at 37 C resulted in complete loss of inhibitory activity. The presence of a ribonuclease in the preparations could conceivably mimic the action of an inhibitor of the enzymes, by diminishing the macromolecular RNA remaining for the final precipitation. To rule out such a possibility, 14C-uracil-labeled tRNA, isolated from E. coli, was incubated with various amounts of the preparations containing the putative inhibitor under the conditions of enzyme assay. The recovery of radioactive RNA from these incubations was comparable to those from controls, which received no supernatant fraction.

The possibility of hydrolysis of S-adenosylmethionine by the supernatant fraction during incubation was also considered. However, additional amounts of S-adenosylmethionine at 10 and 20 min during 30 min of incubation had no effect on the extent of inhibition by the supernatant fraction. That the inhibition was due to the destruction of the methylases during incubation under the influence of a protease was also ruled out by the following experiment. The pH 5.0 supernatant fraction was added to the pH 5.0 precipitate, and the mixture was incubated at 37 C for 30 min. The pH 5 precipitation was repeated; the recovery of enzyme activity in the precipitate was comparable to that of a control, which was incubated without supernatant fluid.
and which received supernatant fluid just prior to pH 5.0 precipitation.

Specificity of the inhibitor. The inhibitor from 8-hr aggregated amoebae inhibited the methylases from free amoebae, 4-hr aggregated amoebae, and the enzymes from spores. The inhibitor from the spores also had a wide range of activity, inhibiting the methylases from both the free and the aggregated amoebae. The inhibitor from 8-hr aggregated amoebae was also active against tRNA methylase from A. aerogenes and from E. coli (tested with methyl-deficient tRNA). The inhibitor, therefore, apparently is not species specific.

DISCUSSION

Naturally occurring inhibitors of tRNA methylases have been observed in other biological systems; in induced lysogenic organism, a specific dialyzable inhibitor of uracil methylase appears (17). S. J. Kerr from this laboratory has isolated an inhibitor of protein nature from adult mamalian liver and brain (Fed. Proc., p. 866, 1969).

Control mechanisms at the molecular level, which trigger morphogenesis in the slime mold, are obscure. The only agent known to be involved in the process is cyclic adenosine monophosphate, whose presence initiates aggregation of the amoebae (9). Changes in the slime mold's endowment of enzymes during morphogenesis are known (14).

The findings described in an earlier (10) and in this communication imply an alteration in the tRNA forms commencing sometime between the 4th and 8th hr of aggregation. Any newly formed tRNA after this period could be expected to be methylated differently from the tRNA species in the free amoebae.

Some of the functions of the alterations of tRNA forms are known. It was shown by Shugart et al. that the methylation of tRNA of E. coli serves some cognitive role in the interaction with amino acyl synthetases (11). The modification of the base adjacent to the anticodon appears to play some function in ribosomal attachment (7). Certain tRNA forms have been shown to have a regulatory function for protein synthesis in E. coli, as initiators (6) and terminators (12).

More recently, convincing evidence for translational control by tRNA in the synthesis of hemoglobin has been adduced by Anderson and Gilbert (1). Alteration of the tRNA methylases by inhibitors in the differentiating slime mold may also serve a regulatory function in providing specific key tRNA forms for new protein synthesis, or by rendering some tRNA forms nonfunctional for old protein synthesis.

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LITERATURE CITED