Effect of Sclerin and 2,4-Dinitrophenol on Formation of Aminoacyl-tRNA by Mouse Liver Enzyme

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Sclerin (SCL) enhanced the formation of aminoacyl-tRNA by mouse liver enzyme both in a heterologous reaction with yeast tRNA and in a homologous reaction with mouse liver "pH 5 fraction" to which was added CTP. The effect of SCL has been found to involve the following two points of action; one is the stimulation of the formation of aminoacyl-AMP accompanied with the activation of inorganic pyrophosphatase activity in "pH 5 fraction," and the other is an increase in the incorporation of AMP and CMP into the tRNA fraction. On the other hand, 2,4-dinitrophenol (DNP) rather inhibited the pyrophosphatase activity, though its effect on aminoacyl-tRNA formation varied depending on the kind of amino acids and the reaction condition. It is assumed that SCL stimulates amino acid incorporation into the protein and cold TCA soluble fractions of liver tissue through the above-mentioned mechanism of action.

Sclerin (SCL), a physiologically active metabolite produced by a strain of *Sclerotinia libertiana*, stimulates the growth and enzyme formation of various seedlings such as those of castor bean, mung bean, radish, barley and rice,^{1~3)} and especially the growth of sugar beet.⁴⁾ SCL also stimulates the formation of enzymes by microorganisms such as lipase by the *Sclerotinia* fungus itself,¹⁾ and amylase and protease by washed suspensions of a strain of *Bacillus subtilis*.³⁾ SCL may influence widely the regulation of metabolisms in cells of many living organisms, where a common mechanism of action at the enzyme level may operate through plants and animals. In an experiment on animal tissue, 2,4-dinitrophenol (DNP)

competitively repressed the effect of SCL on enzyme induction.³⁾ In this paper, the effect of SCL and DNP on the formation of aminoacyl-tRNA by mouse liver enzyme is presented, and some aspects of the mechanism of SCL action are elucidated.

MATERIALS AND METHODS

Chemicals. Nucleotide triphosphates and yeast-tRNA were purchased from Boehringer Mannheim Japan Co., Ltd. Dehydrostreptomycin sulfate was purchased from Takeda Chemical Industries Ltd. L-¹⁴C-Valine (225 mCi/mM), L-¹⁴C-isoleucine (270 mCi/mM), L-¹⁴C-phenylalanine (405 mCi/mM), L-¹⁴C-methionine (5.81 mCi/mM) and 8-¹⁴C-ATP (49.8 mCi/mM) were purchased from Daiichi Pure Chemicals Co., Ltd.

Incorporation of amino acid into liver tissue. Mice (DDT strain), which had been fed with a diet of Clea Co., Ltd. (CE-2) and water and weighed 20 to 30 g each, were killed by decapitation. Livers were removed and put in a chilled dish. It was sliced with a razor blade, and divided into each 100 mg portions. Incubation was carried out in 2 ml of Krebs-Ringer

* To whom requests for reprints should be addressed. Abbreviations. RNA, Ribonucleic acid; tRNA, Transfer RNA; TCA, Trichloroacetic acid; ATP, Adenosine 5'-triphosphate; AMP, Adenosine 5'-phosphate; CTP, Cytidine 5'-triphosphate; CMP, Cytidine 5'-phosphate; P_i, Orthophosphate; PP_i, Pyrophosphate; pC, Cytidine 5'-phosphate; Cp, Cytidine 3'-phosphate; A, Adenosine; *K_m*, Michaelis constant.

solution containing 2 mg of glucose, 2 mg of casamino acids and 6.2×10^{-5} mCi of ^{14}C -U-labeled amino acids for 60 min at 37°C with shaking. After the incubation the slices were washed and homogenized, and the homogenate was fractionated by a modified method of Schmidt-Thanhauser-Schneider.^{5,61} Radioactivity was counted by an Aloka FC-1E gas flow counter with a TDC-2 autoscaler.

Aminoacylation of tRNA in homologous and heterologous reactions. In the homologous reaction, the mouse liver "pH 5 enzyme" fraction was used. The "pH 5 fraction" was prepared from the $105,000 \times g$ supernatant fraction of the liver homogenate, and 0.2 ml of the pH 5 fraction was preincubated with 3 μmoles of ATP, 4.5 μmoles of MgCl_2 and 30 μmoles of Tris buffer (pH 7.7) for 30 min at 37°C in the presence or absence of 0.4 μmole of CTP. The preincubated solution was again incubated with 0.2 ml of the "pH 5 fraction," 3 μmoles of ATP, 4.5 μmoles of MgCl_2 , 30 μmoles of Tris buffer and 0.2 μmoles of ^{14}C -U-labeled amino acid for 30 min at 37°C ; the total volume of the incubated mixture was 1.1 ml. In the heterologous reaction, the mouse liver "streptomycin supernatant fraction" prepared according to the method of Daniel and Littauer⁷¹ was used, and 2 mg of yeast tRNA was preincubated with 3 μmoles of ATP, 4.5 μmoles of MgCl_2 , 30 μmoles of Tris buffer (pH 7.7) and 0.1 ml of the "streptomycin supernatant fraction" for 30 min at 37°C . To the preincubated solution were added 0.2 μmole of ^{14}C -U-labeled amino acid (6×10^4 cpm), 3.0 μmoles of ATP, 4.5 μmoles of MgCl_2 , 30 μmoles of Tris buffer (pH 7.7) and 0.1 ml of the "streptomycin supernatant fraction," and the mixture (1.0 ml) was again incubated for 30 min at 37°C . After the incubation, 27 μmoles of EDTA and 1.0 ml of 70% phenol were added. The labeled aminoacyl-tRNA was separated by the phenol-water procedure and precipitated by the ethanol-salt precipitation. The precipitate was dissolved into 1.0 ml of 0.1% sodium chloride solution, and 0.2 ml of the solution were placed on a stainless steel planchette, evaporated to dryness and counted by an Aloka FC-1E 2 gas flow counter.

Incorporation of AMP into tRNA. The extent of AMP incorporation was measured as follows: A reaction mixture (0.45 ml) containing 20 μmoles of glycine buffer (pH 9.4), 0.3 μmole of 8- ^{14}C -labeled ATP (2×10^4 cpm), 0.3 μmole of unlabeled CTP, 2.0 μmoles of MgCl_2 , 5.0 μmoles of phosphoenolpyruvate, 0.02 ml

of pyruvate kinase (2.0 μg of protein), 0.15 mg of yeast tRNA and 0.1 ml of the "streptomycin supernatant fraction" was incubated at 37°C for an appropriate time. The reaction was stopped by immersing the tubes in an ice bath, and 0.5 ml of 5% perchloric acid was added. The resulting precipitate was filtered through a millipore filter (0.45 μ), and washed three times with 5 ml portions of 1% perchloric acid. Then, the filter was transferred to a planchette, dried and counted by the gas flow counter.

MAK column chromatography. Methylated albumin was prepared as described by Mandell and Hershey.⁸¹ Column chromatography was performed according to the method of Sueoka and Yamane,⁹¹ and ^{14}C -labeled aminoacyl-tRNA (1.2 mg) in 50 ml was loaded on a 2.0×17.2 cm column equilibrated with 0.4 M NaCl and 0.2 M phosphate buffer (pH 6.7). Fractions (5 ml) were collected at a flow rate of 40 ml/hr.

Formation of hydroxamic acid from aminoacyl-AMP. The method of Lipmann and Tattle¹⁰¹ was followed. A mixture (1 ml) containing 0.3 mg of the "streptomycin supernatant fraction," 1.2 mmoles of salt free hydroxylamine, 10 μmoles of disodium ATP, 10 μmoles of phenylalanine and 60 μmoles of Tris buffer (pH 7.8) was incubated at 37°C for an appropriate time. At the end of the incubation, it was cooled to 0°C and mixed with 1 ml of a ferric chloride solution (5 per cent in 0.1 N hydrochloric acid). The resulting precipitate was removed by centrifugation. The absorption of the supernatant fluid at 540 nm was measured against a blank containing all the components except amino acid.

Measurement of pyrophosphatase activity. A reaction mixture containing 0.33 mole of pyrophosphate, 1.7 mmoles of MgCl_2 , 0.3 ml of the "pH 5 fraction" and 0.33 mole of Tris buffer (pH 7.7) in the total volume of 3.3 ml was incubated for 30 min at 37°C , and 3 ml of 5% TCA was added. Amount of P_i released was determined by the method of Fiske-Subbarow.¹¹¹

RESULTS

1. Effect of SCL and DNP on amino acid incorporation into liver tissue

As shown in Table I, SCL (2×10^{-5} M) stimulated both the incorporation of L-valine and L-phenylalanine into the protein fraction and

TABLE I. EFFECT OF SCL AND DNP ON ^{14}C -AMINO ACIDS INCORPORATION INTO LIVER TISSUE

		Amount of amino acid incorporated, cpm/100 mg tissues			
		Control	SCL ^{a)}	DNP ^{b)}	SCL ^{a)} + DNP ^{b)}
Protein fraction	{L- ^{14}C -Valine	178	195	163	109
	{L- ^{14}C -Phenylalanine	355	376	206	239
Cold TCA soluble fraction	{L- ^{14}C -Valine	325	486	497	201
	{L- ^{14}C -Phenylalanine	402	524	289	720

^{a)} SCL at 2×10^{-5} M.^{b)} DNP at 1.72×10^{-5} M.

cold TCA soluble fraction of liver tissue, respectively. DNP (1.72×10^{-5} M) inhibited the incorporation of these amino acids into the protein fraction. Although it inhibited the incorporation of phenylalanine into the cold TCA soluble fraction, it rather stimulated that of valine. The combination of SCL and DNP also inhibited the incorporation of the amino acids into the protein fraction. Although it inhibited the incorporation of valine into the cold TCA soluble fraction, it rather markedly stimulated that of phenylalanine.

2. Effect of SCL and DNP on formation of aminoacyl-tRNA in heterologous reaction with yeast tRNA

As shown in Table II, SCL more or less enhanced the formation of all the aminoacyl-tRNAs tested such as those of valyl-, phenylalanyl-, isoleucyl-, and methionyl-tRNA. DNP slightly stimulated the formation of valyl-, and phenylalanyl-tRNAs while it completely inhibited that of isoleucyl and methionyl-tRNAs. The effect of combination of SCL and DNP on the formation of aminoacyl-tRNAs varied depending on the kind of amino acids.

The effect of concentration of SCL on the formation of phenylalanyl-tRNA was presented in Fig. 1. The effect of SCL increased with the concentration and was maximum at 4×10^{-5} M. The activity of the mouse liver enzyme on the formation of phenylalanyl-tRNA was fairly high as compared with that of the rat liver enzyme,¹²⁾ in the heterologous

TABLE II. EFFECT OF SCL AND DNP ON AMINOACYLATION OF tRNA IN HETEROLOGOUS REACTION

	Amount of amino acid esterified, $\mu\text{moles/mg RNA}$			
	Control	SCL ^{a)}	DNP ^{b)}	SCL ^{a)} + DNP ^{b)}
L- ^{14}C -Valine	0.125	0.208	0.145	0.371
L- ^{14}C -Phenylalanine	0.608	0.745	0.712	0.434
L- ^{14}C -Isoleucine	0.002	0.027	0.00	0.00
L- ^{14}C -Methionine	0.001	0.0125	0.00	0.151

^{a)} SCL at 2×10^{-5} M.^{b)} DNP at 1.72×10^{-5} M.

FIG. 1. Effect of Concentration of SCL on Formation of Phenylalanyl-tRNA.

reaction with yeast tRNA. As presented in Fig. 2, MAK column chromatography of ^{14}C -valine-tRNAs formed in the presence or absence of SCL was carried out. While two peaks were observed in the absence of SCL, only one peak was observed in the presence

FIG. 2. MAK Column Chromatography of ^{14}C -Valine-tRNA.
A, ^{14}C -valine-tRNA formed in the absence of SCL.
B, ^{14}C -valine-tRNA formed in the presence of SCL
($2 \times 10^{-5} \text{ M}$).
The procedure is described in the text.

of SCL. The main peak of the former was usually eluted at tube No. 25 to 45 and the peak of the latter was eluted at tube No. 38 to 50 with high radioactivity. These results suggest that SCL may cause a change in the molecular structure of tRNA during the incubation with the mouse liver enzyme.

3. Effect of SCL on formation of aminoacyl-AMP

Aminoacyl-AMP was trapped with hydroxylamine, and the amino-hydroxamic acid formed was determined by colorimetry as described above. The time course for the formation of phenylalanine-hydroxamic acid in the presence or absence of SCL was presented in Fig. 3. SCL at a concentration of $1 \times 10^{-4} \text{ M}$ distinctly stimulated the formation of phenylalanine-hydroxamic acid from the beginning for 60 min.

FIG. 3. Time Course of Amino-Hydroxamic Acid Formation.
○---○ SCL added at $1 \times 10^{-4} \text{ M}$; ●---● control.
Phenylalanine was used.

4. Effect of SCL and DNP on pyrophosphatase activity in "pH 5 fraction"

As shown in Table III, pyrophosphatase

activity in the "pH 5 fraction" was stimulated by SCL ($2 \times 10^{-5} \text{ M}$), but it was inhibited by DNP ($1.72 \times 10^{-5} \text{ M}$) and also by a combination of SCL and DNP. The effect of the concen-

TABLE III. EFFECT OF SCL AND DNP ON PYROPHOSPHATASE ACTIVITY IN "pH 5 Fraction"

Control	P _i released (mμmole)		
	SCL ^{a)}	DNP ^{b)}	SCL ^{a)} +DNP ^{b)}
5.2	6.4	4.32	4.32

^{a)} SCL at 2×10^{-5} M.^{b)} DNP at 1.72×10^{-5} M.

FIG. 4. Effect of Concentration of SCL on Pyrophosphatase Activity in "pH 5 Fraction."

FIG. 5. Lineweaver-Burk Plot for Effect of SCL and DNP on Pyrophosphatase Activity in "pH 5 Fraction."

○—○ Control; ●—● SCL (2×10^{-5} M); ×—× DNP (1.72×10^{-5} M).

tration of SCL on pyrophosphatase activity in the "pH 5 fraction" was presented in Fig. 4. The effect of SCL was maximum at a concentration of 2×10^{-5} M. The Lineweaver-

Burk plot for the effect of SCL and DNP on pyrophosphatase activity was presented in Fig. 5. SCL had an effect of lowering the K_m value, and DNP showed a mixed type of inhibition.

5. Effect of SCL on aminoacylation of tRNA in the presence of pyrophosphate

As shown in Table IV, while the formation of aminoacyl-tRNAs in the presence of pyrophosphate at a concentration of 10^{-6} M was completely inhibited, it was restored by SCL at a concentration of 2×10^{-5} M both in the heterologous and homologous reactions.

TABLE IV. EFFECT OF SCL ON AMINOACYLATION OF tRNA IN THE PRESENCE OF PYROPHOSPHATE

Formation of 14 C-valine-tRNA in the presence of PP_i at 10^{-6} M was determined as described in the text.

	Amount of amino acid esterified, (mμmoles/mg RNA)	
	Control	SCL (2×10^{-5} M)
Heterologous reaction	0.00	1.34
Homologous reaction	0.00	0.16

6. Effect of SCL and DNP on aminoacylation of tRNA with homologous reaction in the presence or absence of CTP

As shown in Table V, in the homologous reaction DNP (1.72×10^{-5} M) rather stimulated the aminoacylation of tRNA in the absence of CTP, while SCL (2×10^{-5} M) did not. On the other hand, SCL markedly stimulated the aminoacylation of tRNA in the presence of CTP, while DNP inhibited conversely. The effect of a combination of SCL and DNP was also inhibitory.

7. Effect of SCL and CTP on AMP incorporation into tRNA

As shown in Table VI, the effect of SCL and CTP on the incorporation of AMP from

TABLE V. EFFECT OF SCL AND DNP ON AMINOACYLATION OF tRNA WITH HOMOLOGOUS REACTION IN THE PRESENCE OF CTP

Formation of ^{14}C -valine-tRNA with the homologous reaction in the presence or absence of CTP was determined as described in the text.

	Amount of amino acid esterified, (m μ moles/mg RNA)			
	Control	SCL ^{a)}	DNP ^{b)}	SCL ^{a)} +DNP ^{b)}
None	0.24	0.20	0.51	0.23
CTP ^{c)}	0.40	1.61	0.05	0.10

^{a)} SCL at 2×10^{-5} M.

^{b)} DNP at 1.72×10^{-5} M.

^{c)} CTP, in 0.4 μ mole.

TABLE VI. EFFECT OF SCL AND CTP ON AMP INCORPORATION INTO tRNA

	Amount of AMP incorporated in 20 min, m μ moles/mg RNA			
	in the absence of CTP ^{a)}		in the presence of CTP ^{a)}	
	Control	SCL ^{b)}	Control	SCL ^{b)}
Preincubated yeast tRNA ^{c)}	3.10	4.20	3.70	5.20
Untreated yeast tRNA	6.10	6.80	6.20	8.16

^{a)} CTP at 3×10^{-7} M.

^{b)} SCL at 2×10^{-5} M.

^{c)} Yeast tRNA, 5 mg was preincubated with 1 ml of the "streptomycin supernatant fraction" and 1 ml of Tris buffer (pH 7.7) for 2 hr at 37°C, and separated by the phenol method. The conditions are described in the text.

ATP into yeast tRNA was examined. SCL (2×10^{-5} M) only increased remarkably the rate of AMP incorporation in the presence of CTP (3×10^{-7} M) in 20 min. However, when yeast tRNA was preincubated with the "streptomycin supernatant fraction," SCL remarkably increased the rate of AMP incorporation both in the presence and absence of CTP. Also, as presented in Fig. 6, AMP incorporation into preincubated tRNA in the absence of CTP was stimulated by SCL at the beginning, but the stimulation did not extend further

FIG. 6. Time Course of Incorporation of AMP into Preincubated Yeast tRNA.

●—● Control } in presence of CTP
●---● SCL at 2×10^{-5} M } (3×10^{-7} M)
○—○ Control } in absence of CTP
○---○ SCL at 2×10^{-5} M }

Yeast tRNA was preincubated with the "streptomycin supernatant fraction" as in Table VI.

and attained to an equilibrium within 60 min. On the other hand, in the presence of CTP, the extent of AMP incorporation was finally increased by SCL. These results indicate that SCL stimulates the incorporation into RNA of nucleotides such as AMP and CMP which construct the -pCpCpA end of tRNA.

DISCUSSION

Cantoni¹³⁾ has already found that yeast pyrophosphatase enhances the formation of serine-tRNA (*E. coli*) by a highly purified enzyme from yeast in a manner analogous to macromolecular "enhancing factor" in yeast. According to Jacobson,¹⁴⁾ the fractionation procedure of the "enhancing factor" is, indeed, appropriate for pyrophosphatase, too. Inorganic pyrophosphate would be a potent inhibitor of the formation of aminoacyl-tRNAs in the heterologous reaction. It is interesting that SCL stimulates the formation of aminoacyl-tRNAs with a simultaneous increase in the activity of pyrophosphatase in the mouse liver "pH 5 fraction." The effect of SCL may

partly be due to a prevention of inhibition by pyrophosphate. SCL lowers the K_m value of pyrophosphatase, although DNP shows a mixed type of inhibition, and DNP does not have a unifying effect on the formation of aminoacyl-tRNAs under the experimental conditions. SCL stimulates the reaction velocity in the time course of the formation of aminoacyl-AMP, from the beginning, where the effect of pyrophosphate may be negligible. Accordingly, it is conceivable that SCL and DNP have a close connection with the specificity of aminoacyl-tRNA synthetase *per se*, respectively.

It is known that, in the heterologous reaction, mischarging of tRNA occurs under various conditions.¹⁵⁾ As shown in the above experiment, in the absence of SCL, two chromatographically distinct ¹⁴C-valine-tRNAs have been formed from a yeast tRNA preparation, while, in the presence of SCL, only one identical ¹⁴C-valine-tRNA separated by a column chromatography. SCL may prevent the mischarging of tRNA by arranging the molecular structure of tRNA. tRNA chains, particularly in yeast tRNA preparations, predominantly terminates in cytidine,¹⁶⁾ and preincubation of yeast tRNA with the "streptomycin supernatant fraction" might remove the terminal cytidine. SCL stimulates the incorporation of AMP and CMP into tRNA, as described above. Therefore, SCL must enhance the extent of the formation of aminoacyl-tRNAs by constructing promptly the -pCpCpA end of tRNA.

SCL facilitates amino acid incorporation into the protein fraction of liver tissue, while DNP inhibits, as described above. There may be involved a still unknown effect in the

protein synthesis. However, it seems reasonable to assume that the main effect of SCL on the protein synthesis is involved in the formation of aminoacyl-tRNAs.

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