SENSITIVITIES OF INCORPORATION OF AMINO ACIDS INTO RIBOSOMAL AND NON-RIBOSOMAL PROTEINS TO VARIOUS ANTIBIOTICS

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Recent publications from various laboratories indicate that synthesis of certain proteins are refractory to the action of inhibitors of general protein synthesis such as puromycin and chloramphenicol[1-6].

Ribosomes are known to participate in protein synthesis and consist of about 57 ribosomal proteins. Although some of the genes for these proteins have been characterized and shown to be clustered[7,8].

This communication deals with amino acid incorporation into ribosomal proteins and their sensitivity to various inhibitors in comparison with non-ribosomal proteins. Furthemore, we have compared synthesis of ribosomal and non-ribosomal proteins in three different temperature sensitive mutants of *E. coli* defective in the elongation factor C_1[9], valyl tRNA sythetase and phenylalanyl tRNA synthetases.

MATERIALS AND METHODS

Bacterial Mutants and Materials. The temperature sensitive mutant defective in phenylalanyl tRNA synthetase (NP 37) and its wild type (NP 3) were grown in medium (composed of 0.1 mM Magnesium acetate-60 mM KCl-6 mM β-mercaptoethanol-10 mM Tris-HCl (pH 7.8)[10] supplemented with thiamine (1 g/ml). The valyl tRNA synthetase(defective) temperature sensitive mutant was grown in medium supplemented with thymidine 40 μc/ml. The t_s mutant with elongation factor C_1 (EFG), *E. coli* B was grown in medium supplemented with methionine 30 μc/ml.

Chloramphenicol(CM), tetracycline hydrochloride (TC) and guanosine triphosphate trisodium (GTP) were purchased from Calbiochem(Calbiochem. Ltd., USA) : Streptomycin sulfate (SM) and puromycin dihydrochloride (PM) were from Nutritional Biochemical Corporation (Cleveland, Ohio) : Erythromycin-ilotycin glycerate (EM) from Eli Lilly and Co. (Indianapolis, Indiana) : Deoxyribonuclease from Worthington Biochemical Co. (Freehold, N. J.) : Lysozyme from Schwartz Mann Research (Orangeburg, N. Y.) : Radioactive Amino Acids were purchased from New England Nuclear Co. (Boston, Mass.).

Amino acid incorporation at the non-permissive temperature. Cells were grown overnight at the permissive temperature in the presence of 3 μc ^{14}C-arginine (272 μc/moles) in 70 ml of a minimum medium[10] with 0.5% glycerol as a carbon source. When the culture reached permissive temperature for 5 minutes, 10 μc of ^{3}H-arginine (528 μc/μ mole or 1.23 C/m mole) were added, and the culture was then incubated for an additional 5 minutes at the non-permissive temperature. In a similar manner, the other 10 ml portion was exposed to ^{3}H-arginine at the permissive temperature for 5 minutes. Cells were harvested after the addition of crushed ice[10] and treated by the FLESSEL's polysome isolation procedure[11] to separate ribosomes from soluble proteins. Nascent peptides attached to polysomes were removed by the treatment with 5×10^{-4} M puromycin. Ribosomes were further washed with Tris-HCl buffer containing 0.5 M NH_4Cl to insure removal of nascent peptides[10].

Chromatography of ribosomal proteins. Comparison of the chromatographic behavior of 30 S ribosomal proteins derived from these mutants treated at either the permissive or non-permissive temperature was carried out as follows : Cells were grown at the permissive temperature in 140 ml growth medium to mid-logarithmic phase and divided into two equal proteins. One portion was incubated for 5 minutes at the non-permissive temperature, then 1 mc of ^{3}H-arginine was added, and further incubated at the non-permissive temperature for 5 minutes. The remaining culture was shaken in the presence of 10 μc of ^{14}C-arginine at permissive temperature for 5 minutes. Ribosomal

proteins prepared from these two cultures were mixed and cochromatographed on phosphocellulose column[12] (1.5×60 cm) using a linear gradient of 0～0.6 M NaCl in 1200 ml of 6 M Urea containing 0.012 M methylamine and 0.05 M NaH_2PO_4 (pH 6.0) at a flow rate of 3.3 ml/10 minutes[10]. Each eluted fraction was filtered through a millipore filter after precipitation with 10% trichloracetic acid[13].

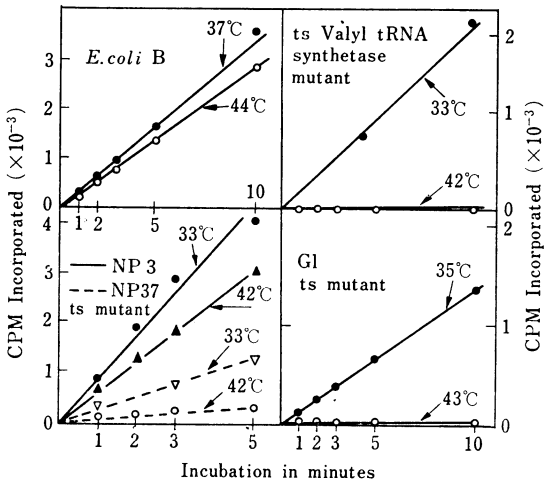
The sensitivity of amino acid incorporation into ribosomal proteins to known inhibitors of protein synthesis such as tetracycline, erythromycin, streptomycin and chloramphenicol were determined by using 70 ml of cell suspension of *E. coli* B. Cells were labeled overnight with 3 μc of ^{14}C-arginine at 37°C. When cell density reached 0.1～0.3 at 550 nm, 10 μc ^{3}H-arginine was added to 10 ml of the culture with the antibiotics and incubated for 5 minutes. Control cultures were labeled in the absence of antibiotics with ^{3}H-arginine for 5 minutes.

RESULTS AND DISCUSSION

Effect of non-permissive temperature and various antibiotics on the amino acid incorporation by *E. coli*. Before undertaking studies on the relative sensitivity of amino acid incorporation into ribosomal and soluble proteins, it was necessary to determine the effect of antibiotics and non-permissive temperature on the general protein synthesis of *E. coli*. *E. coli* mutant strain G_1 has a temperature sensitive elongation factor G and is therefore unable to grow at non-permissive temperature (40°C). Similarly *E. coli* thermosensitive mutants NP 29 and NP 37 have genetic block at valyl and phenylalanyl tRNA synthetase, respectively. Data indicated in Fig. 1 showed that rapid and effective inhibition of amino acid incorporation takes place by either temperature shift (Fig. 1 A) or addition of inhibitors (Fig. 1 B). It should be pointed out that residual incorporation of amino acids either in the presence of inhibitor or at non-permissive temperature proceeded linearly as time progressed. There was no indication of initial incorporation of amino acids immediately after the addition of inhibitors followed by tapering off amino acid incorporation later. The longer the time of theassembly the less insensitivity to any inhibitor one should expect under such conditions. Although the kinetics of residual amino acid incorporation into the ribosomal proteins in the ribosomes was not determined. one can perhaps assume that this too would be

Fig. 1. Effect of non-permissive temperature and various antibiotics on the amino acid incorporation by *E. coli*

A. Bacterial cells 1～2 ml having optical density 0.1～0.15/ml at 550 nm were incubated 5 min. at non-permissive temperature as indicated in the figure before the incubation of cells with 2.5 μc of ^{14}C-alanine (12 μc/μmoles) or 1 μc of ^{14}C-arginine for further incubation. For control culture preincubation at non-permissive temperature was omitted. Aliquots of 0.1 ml were taken at various time intervals and processed for measurement of hot trichloroacetic acid (TCA) insoluble radioactivity as described[13].



B. Experimental conditions were the same as above except *E. coli* B was grown to 0.1～0.3 OD at 550 nm at 37°C and incubated in the presence of various antibiotics as indicated in the figure. Control culture was not added with inhibitors.

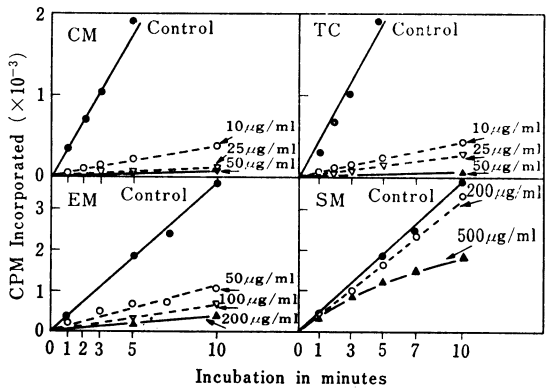


Table 1. Effect of incubation at non-permissive temperature on the incorporation of ^{3}H-arginine into soluble and ribosomal proteins by the temperature sensitive mutants

Bacterial strains	Temperature shift		$^{3}H/^{14}C$ Ratio as percent of control	
			Soluble fraction	Ribosomal fraction
E. coli B	37°C	44°C	86.4	58.6
NP 3	33°C	42°C	76.0	75.3
NP 37	33°C	42°C	11.3	54.0
Valyl tRNA synthetase mutant	33°C	42°C	7.0	16.8
CIB	35°C	43°C	0	16.6

Experimental conditions were as described in Methods.
NP 3—wild type parent of the phenylalanyl tRNA temperature sensitive mutant.
NP 37—derivative of NP 3 possessing a phenylalanyl tRNA temperature sensitive mutant.
GIB—translocase factor G 1 temperature sensitive mutant.

also linear.

Effect of the non-permissive temperature on the incorporation of ^{3}H-arginine into soluble and ribosomal proteins. In the experiment shown in Table 1, cells prelabeled with ^{14}C-arginine for many generations were labeled with ^{3}H-arginine either at non-permissive or permissive temperature and ribosomes were separated from soluble fractions. With precautions to remove all the nascent peptidyl tRNA on the ribosomes, the labeled ribosomal proteins were isolated and compared into amino acid incorporation with the soluble proteins. Comparing with that in soluble proteins relative high radioactivity of ^{3}H both at permissive and at non-permissive temperature in ribosomal proteins were observed. The $^{3}H/^{14}C$ ratios for these fractions thus obtained are shown in Table 1. It is noted that the incorporation of labeled amino acids into soluble and the ribosomal proteins by wild type *E. coli* B were slowed down at 44°C. This effect was more pronounced on ribosomal proteins. On the other hand, in the case of another wild type *E. coli* NP 3, no appreciable difference was obtained on amino acid incorporation into the two different proteins. With mutants having thermosensitive amino acyl tRNA synthetase, a significantly decreased incorporation into soluble proteins was observed than that into ribosomal proteins. In the similar manner, a significant residual activity for amino acid incorporation into ribosomal protein by G 1 B mutant with thermolabile EFG was observed under the condition where inhibited completely the incorporation into the soluble fraction.

Effect of various antibiotics on the incorporation of amino acid into soluble and ribosomal proteins.

Table 2. Comparison of effect of various antibiotics on the incorporation of radioactive arginine into ribosomal and soluble proteins

	Antibiotics	$^{3}H/^{14}C$ Ratio as percent of control value	
		Soluble protein	Ribosomal protein
	None	100	100
C M	10 μg/ml	6.42	20.2
	25 μg/ml	4.12	9.16
	50 μg/ml	0.25	4.97
T C	10 μg/ml	7.25	13.2
	25 μg/ml	2.91	9.32
	50 μg/ml	0	2.43
S M	200 μg/ml	73.0	120.8
	500 μg/ml	32.2	73.3
E M	50 μg/ml	21.85	32.5
	100 μg/ml	13.58	23.5
	200 μg/ml	9.15	17.42

Experimental conditions are as described in Methods.

Relative sensitivity to antibiotics of ribosomal and soluble proteins in the amino acid incorporation was studied as shown in Table 2. In these experiments, various antibiotics that work as protein synthesis inhibitors were added to the growing *E. coli* culture which had been prelabeled with ^{14}C-arginine for many generations. ^{3}H-arginine was added simultaneously with the addition of antibiotics. The ratio of $^{3}H/^{14}C$ in the soluble proteins and in the ribosomal proteins were compared in the presence and absence of inhibitors. It can be concluded from the Table 2 that incorporation of amino acids into the soluble proteins were more sensitive to antibiotics than ribosomal

proteins. Thus, only 0.25% of residual incorporation of amino acids into soluble protein was observed in the presence of chloramphenicol 50 μg/ml, whereas significant amount of residual incorporation into ribosomal proteins remained. Similar relative insensitivity of ribosomal proteins to various antibiotics was also observed. There appeared to be at least two possible interpretations for these results. The first is that some of the ribosomal protein synthesis may be refractory to conventional protein synthesis inhibitors. Similar tendency was observed when mutants having genetics block at some stage of protein synthesis were tested for their relative sensitivity for the amino acid incorporation into soluble and ribosomal proteins. The second, it may be that there are two different systems between soluble and ribosomal protein synthesis.

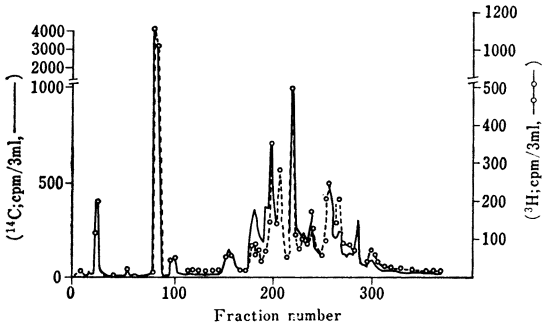
Chromatographic separation of ribosomal proteins. Since there are about fifty-seven ribosomal proteins, preliminary separation of 30 S ribosomal proteins on phosphocellulose column was performed to study relative sensitivities of amino acid incorporation into various 30 S ribosomal proteins. As shown in Fig. 2, the inhibitory effect of non-permissive temperature was not uniform among 30 S ribosomal proteins. In this experiment, cells were exposed to ^{14}C-arginine for many generations and ^{3}H-arginine was given at the non-permissive temperature. The ^{3}H-radioactivity incorporated at the non-permissive temperature did not distribute proportionately among the ribosomal proteins, resulting in the variable ratios of ^{3}H/^{14}C between the peaks of ribosomal proteins. Using mutants whose thermosensitive block is at the step of the valyl or phenylalanyl tRNA synthetase, incorporation of arginine was studed. The effect of non-permissive temperature on the incorporation of arginine is not directly on the arginine incorporation but caused indirectly by the depletion of valyl or phenylalanyl tRNA at the non-permissive temperature.

There have been numerous reports that synthesis of certain proteins are relatively insensitive to certain antibiotics[1-6] suggesting some proteins may be synthesized in a specific system which is different from that of cytoplasmic protein synthesis.

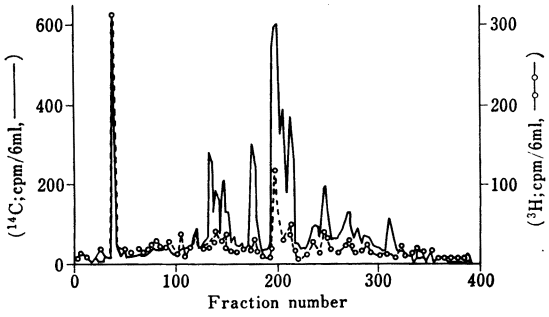
Our present observations are consistent with the notion that synthesis of ribosomal proteins may be refractory to certain inhibitors of protein synthesis. Similar insensitivity was observed at the non-permissive temperature of thermosensitive mutant having a genetic block at step of protein biosynthesis. It should be emphasized that labeled amino acid incorporation into certain proteins in cell organell may not necessarily reflect the relative sensitivity of the synthesis of that particular protein. If the inhibitory effect is not complete (usually residual activity exists) and the residual activity in the presence of inhibitors tapers off as the time of incubation proceeds, one may expect

Fig. 2. Chromatographic profiles of 30 S ribosomal proteins isolated from phenylalanyl tRNA and valyl tRNA synthetase temperature sensitive mutants

A. Culture of temperature sensitive mutant of phenylalanyl tRNA synthetase 70 ml having OD 0.1 at 550 nm was shaken at 33°C in the presence of 20 μc ^{14}C-arginine. The other 70 ml was shaken at 42°C for 5 min. and further incubated with 1 mc of ^{3}H-arginine for 5 min. Ribosomal proteins of 30 S subunits were isolated from these labeled cells as described in the text and isolated proteins with radioactivity ^{14}C-6.3×10^{4} cpm and ^{3}H-3.3×10^{4} cpm in 143 OD at 230 nm were chromatographed as described in the Materials and Methods.



B. Same as A except 10 μc of ^{14}C-arginine was used for labeling of protein at permissive temperature of Valyl tRNA synthetase temperature sensitive mutant.



apparent relative insensitivity of any protein organell. This is due to time necessary for assembly of the organell. If, however, the residual activity persists without tapering off in a linear fashion, incorporation of amino acids into organell proteins should be equally sensitive to inhibitors unless there are basic differences in the mechanism of synthesis of the protein. To determine these two possibilities, one would have to study the kinetics of inhibition of amino acid incorporation into ribosomal proteins as compared to soluble proteins. Although these kind of studies were not performed in the present communication, the kinetics of inhibition on the over all protein synthesis was studied. Except for the case with streptomycin, the inhibitory effect was observed very rapidly and the residual activity persisted linearly and there was no sign of tapering off.

Non-uniform effect of non-permissive temperature on various ribosomal proteins may indicate that the synthesis of these proteins have different sensitivity to certain antibiotics. However, it is entirely possible that time required for newly synthesized proteins to be incorporated into ribosomal particles may vary depending on the ribosomal proteins. If inhibitory effect on the ribosomal proteins is such that gradual tapering off of synthesis takes place, apparent relative insensitivity of certain ribosomal protein may be observed if the pool size of the particular ribosomal protein was large so that relatively long time is necessary for the newly synthesized proteins to be incorporated into ribosomes. This kind of consideration should apply to all protein synthesis measured in cell organell such as membrane, mitochondria, nuclear histone, *etc.* Whether or not the relative antibiotic insensitivity of amino acid incorporation into ribosomal proteins as compared to that into soluble proteins may relfect possibility that certain ribosomal proteins may have different mechanism for its synthesis remains to be determined[11].

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Summary

Comparative studies of the incorporation of amino acids into ribosomal and non-ribosomal proteins were carried out employing various antibiotics, inhibitors of protein synthesis, as well as mutants of *E. coli* thermosensitive for some stages of protein synthesis.

With mutants having thermosensitive amino acyl tRNA synthetase, a significantly decreased incorporation into soluble protein was observed than that into ribosomal proteins.

It was found that the incorporation of amino acids into ribosomal proteins were less inhibited than that into non-ribosomal proteins by various antibiotics.

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