J. Biochem. 119, 1076-1079 (1996)

Pyridine-Promoted Factor- and Energy-Free Peptide Synthesis Systems Prepared from Various Organisms Including Prokaryote, Eukaryote, and Mitochondria

Takahiko Nojima, Itaru Nitta, Takuya Ueda, and Kimitsuna Watanabe[1]

Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, 7-3-1 Bunkyo-ku, Tokyo 113

Received for publication, December 21, 1995

We demonstrate here that ribosomes from not only *Escherichia coli* and *Thermus thermophilus* [Nitta *et al.* (1994) *J. Biochem.* 115, 803-807; *ibid.*, (1995) 118, 841-849] but also yeast and bovine mitochondria catalyze peptide synthesis promoted by a high concentration of pyridine in the absence of soluble protein factors and chemical energy sources, and compare some characteristic features of the reactions among these organisms. Sensitivities against antibiotics, chloramphenicol and cycloheximide, showed the same tendency to those in the *in vitro* aqueous translation systems of these organisms, suggesting that the basic mechanism for peptide synthesis is the same among these organisms. The optimal concentration of pyridine was centered at 50% for all systems, although the dependencies on the pyridine concentrations and the yields of the products were different from one another. All these systems required Mg^{2+}, and only mitochondrial system showed the extra Mn^{2+}-requirement, which enhanced the yield by several fold. The optimum reaction temperatures coincided closely with the growing temperatures of the organisms except for the mitochondrial system, which showed the highest activity above 80°C. The rationale for these observations remains to be solved.

Key words: antibiotics, Mn^{2+} ion, peptide synthesis system, pyridine, ribosomes.

Although about half a century has passed since almost all proteins in the cells were shown to be synthesized on ribosomes, many problems remain to be solved. Ribosomes form a complex system which consists of RNA and a large number of proteins, but the function of each molecule has not yet been fully clarified. It was once believed that various catalytic activities of ribosomes including that of the peptide bond formation resided in the ribosomal proteins, and the ribosomal RNAs (rRNAs) assisted the protein functions (*1*). However, many attempts to find such a protein function were unsuccessful. Recently, some reports have appeared that suggest the rRNA to be responsible for such functions. Noller and co-workers (*2*) showed that 50S ribosomal subunit from *Thermus aquaticus* retained the peptidyltransferase activity even after deproteinization by extensive phenol extraction, and Schulze and Nierhaus (*3*) demonstrated that the peptidyltransferase activity was exerted by 23S rRNA with only a slight amount of the ribosomal proteins. These findings suggest that only rRNA can catalyze the peptide synthesis if the reaction condition is appropriately controlled.

In the previous papers (*4, 5*), we demonstrated that in the presence of a high concentration of pyridine, ribosomes from *Escherichia coli* or *Thermus thermophilus* can synthesize some oligopeptides without the soluble protein factors and chemical energy sources. The activity remained even after phenol extraction of the *T. thermophilus* ribosomes.

[1] To whom correspondence should be addressed. TEL & FAX: +81-3-5800-6950

In this paper, we have successfully constructed such systems from eukaryotic cells (yeast: *Saccharomyces cerevisiae*) and organelle (bovine mitochondria), and determined the optimum conditions for the reaction, such as concentrations of pyridine and cations, as well as the reaction temperatures. We also investigated effects of antibiotics which inhibit peptidyltransferase activity.

MATERIALS AND METHODS

Materials—Transfer RNA specific for phenylalanine from *E. coli* ($tRNA^{Phe}_{E.\,coli}$), chloramphenicol, and cycloheximide were purchased from Sigma. Pyridine was a product of Wako Chemical (Tokyo) and was treated with molecular sieve before use. L-[^{14}C]Phenylalanine (17.5 GBq/mmol) was obtained from Amersham. Silica-Gel plates for thin-layer chromatography were obtained from Merck. All the other reagents used were of the highest biochemical grade available.

Preparations of Ribosomes from Various Organisms and [^{14}C]Phenylalanyl-$tRNA^{Phe}$ of E. coli—Ribosomes (70S) from *E. coli* A19 and *T. thermophilus* HB27 were prepared according to Speeding (*6*) with minor modifications. [^{14}C]Phenylalanyl-$tRNA^{Phe}$ from *E. coli* ([^{14}C]Phe-$tRNA^{Phe}_{E.\,coli}$) was prepared as described previously (*4*). Yeast (*S. cerevisiae* JCM7255) was grown to the middle log phase at 30°C. Ribosomes from this yeast strain (80S) were prepared by the same method as that used for *E. coli* or *T. thermophilus*, except that quartz sand replaced aluminium oxide for cell squash. Ribosomes (55S) were prepared from

bovine mitochondria by the method of Matthews *et al.* (7).

Activation of Ribosomes Prior to the Peptide Synthesis Reaction—Ribosomes suspended in a buffer containing 20 mM Hepes-KOH (pH 7.5) were incubated for 30 min at 37°C (for *E. coli* and yeast systems) or 65°C (for *T. thermophilus* and mitochondrial systems) before the peptide synthesis reactions were carried out.

Polyphenylalanine Synthesis—Typical reaction mixtures for polyphenylalanine synthesis (total volume; 25 μl) contained the following components; 15 A_{260}/ml of ribosomes, 4,000 cpm of L-[^{14}C]Phe-tRNA$^{Phe}_{E.coli}$, 50% (v/v) pyridine, 120 mM KCl, $MgCl_2$ (15 mM for *E. coli* and *T. thermophils*, 1.2 mM for yeast, and 4 mM for mitochondrial systems), and 3 mM $MnCl_2$ (only for mitochondrial system). Incubation was carried out for 60 min at 37°C (for *E. coli* and yeast systems) or 65°C (for *T. thermophils* and mitochondrial systems). The reaction was quenched by addition of aqueous KOH to a final concentration of 30 mM, and deacylation was performed by incubation at 37°C for 90 min. The reaction mixtures were directly spotted onto the thin-layer plate, which was developed as described previously (*4*). Radioactivities of [^{14}C]phenylalanine and its dimer, trimer, and larger oligomers, and total radioactivities on the TLC plate were quantified by use of a FUJIX BAS-1000 bio-imaging analyzer. The yield of [^{14}C]polyphenylalanine synthesis was defined as [the radioactivities of [^{14}C]oligophenylalanines including dimer and trimer]/[the total radioactivities of the spots on the TLC plate].

Inhibition of Peptide Synthesis by Antibiotics—Ribosomes were incubated with 2.5 mM cycloheximide or 25 mM chloramphenicol at 37°C (for *E. coli* and yeast systems) or 65°C (for *T. thermophilus* and mitochondrial systems) for 30 min in 20 mM Hepes-KOH (pH 7.5). Polyphenylalanine synthesis was carried out with the same concentration of antibiotics and the same temperature as in the pre-incubation. After the reaction, the mixtures were loaded onto Whatmann 3 MM filter and washed twice each with 10% TCA and with ethanol for 15 min, followed by drying. The ^{14}C-radioactivity was measured with a liquid scintillation counter.

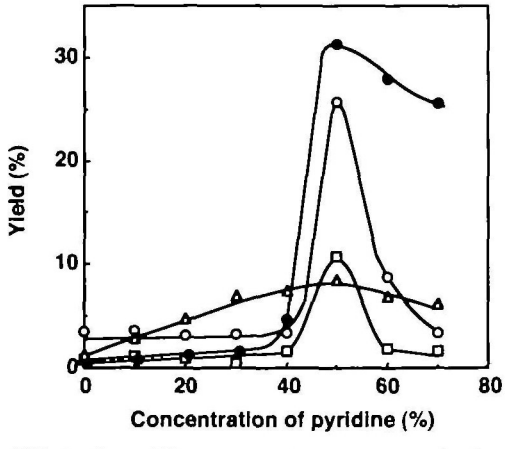

Fig. 1. **Effect of pyridine concentrations on polyphenylalanine synthesis.** Pyridine-promoted factor- and energy-free peptide synthesis systems were constructed from various organisms, *E. coli* (○), *T. thermophilus* (●), yeast (□), and bovine mitochondria (△). The reaction was carried out as described in "MATERIALS AND METHODS."

RESULTS

Comparison of Pyridine-Promoted Factor- and Energy-Free Peptide Synthesis Systems Prepared from E. coli, T. thermophilus, Yeast, and Mitochondria—We have already found that the peptide synthesis reaction can proceed with ribosomes from *E. coli* or *T. thermophilus*, aminoacyl-tRNAs, a template RNA, and Mg^{2+} and K^+ ions in the presence of 40–60% pyridine, where no soluble protein factors or energy sources were required (*4, 5*). At 50% pyridine, the peptide synthesis can occur most efficiently but with no template-dependency.

To ascertain if this is also the case for eukaryotic and organelle translation systems, we used ribosomes from yeast (*S. cerevisiae*) and bovine mitochondria with tRNA from *E. coli*.

Figure 1 shows the relation of the yield of the peptide synthesis with pyridine concentration. As has already been reported (*5*), the *E. coli* system showed a narrow optimal concentration range of pyridine centered at 50%; and a similar spectrum was observed for the yeast system, although the synthesis was less efficient. The *T. thermophilus* system also had a maximum yield at 50% pyridine, but the dependency on pyridine concentration was rather weak in the concentration range higher than 50%. The mitochondrial system had considerably low efficiency and showed a very weak dependency on the pyridine concentration, although the optimal concentration was also 50%. Thus, 50% pyridine was used for all the reactions in this study.

Figure 2 shows the reaction products formed by the pyridine-promoted peptide synthesis systems of *E. coli*, *T. thermophilus*, yeast, and mitochondria. All these systems could produce oligophenylalanines of tetramer and higher forms. The trimer was prominent for *E. coli*, and the dimer was observed for *E. coli*, *T. thermophilus*, and mitochondria; but yeast appeared to produce neither dimer nor

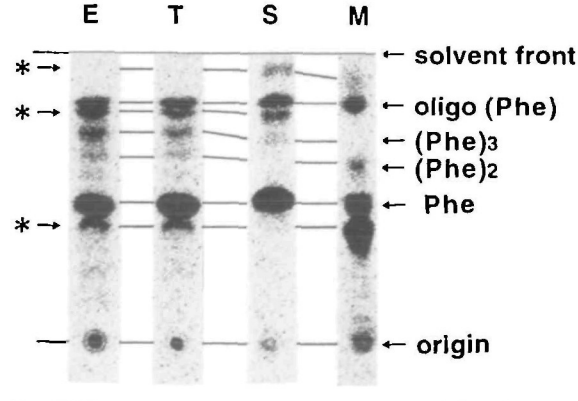

Fig. 2. **TLC analysis of products synthesized by the peptide synthesis system from the various organisms.** Ribosomes were prepared from *E. coli* (E), *T. thermophilus* (T), yeast (S), and bovine mitochondria (M). Reaction was carried out for 60 min at 37°C for *E. coli* and yeast systems or 65°C for *T. thermophilus* and mitochondrial systems. The (oligo)phenylalanine moieties attached to tRNAs were released by alkaline hydrolysis at 37°C for 90 min, and the resultant hydrolysates were developed on the thin-layer plates as described in "MATERIALS AND METHODS." The symbols (*) show unidentified byproducts.

trimer. Unidentified products appear to have been produced in all the systems.

Because the R_f values on the TLC are slightly variable depending on the sample solution including pyridine, the spots including monomer, dimer, trimer, and tetramer of phenylalanine were confirmed by developing with the respective authentic samples in all these four systems. Thus, the corresponding bands are linked with lines between each lane. The spots indicated by stars (*) remain unidentified.

Sensitivity of the Peptide Synthesis Activity toward Antibiotics—To confirm that the peptide synthesis reactions observed for these four different systems as shown in Figs. 1 and 2 are really derived from the peptidyltransferase activity embedded in the ribosomes, sensitivities of the systems toward antibiotics, chloramphenicol and cycloheximide, were examined. It is well known that chloramphenicol inhibits the peptidyltransferase activity in the 50S ribosomal subunit of prokaryotic cells (*8*) as well as in the large subunit of mitochondrial ribosomes (*9*), while cycloheximide inhibits only the peptidyltransferase activity in the 60S ribosomal subunit of eukaryotic cells (*10*).

Table I shows the results. It became clear that chloramphenicol inhibited the peptide synthesis in the *E. coli*, *T. thermophilus*, and mitochondrial systems but not that of the yeast system, whereas cycloheximide inhibited only the peptide synthesis in the yeast system. These results suggest that the pyridine-promoted non-enzymatic peptide synthesis systems described here function in the same manner as the usual aqueous translation systems in the living organisms.

Effect of Cations on the Pyridine-Promoted Peptide Synthesis—Figure 3 shows the dependence of the yield on the concentration of $MgCl_2$. A broad spectrum of Mg^{2+}-dependency was observed for the *E. coli* system, as already reported (*5*), and the *T. thermophilus* system also showed the similar spectrum; whereas in the yeast system 1.2 mM Mg^{2+} was the optimum concentration, and the yield dropped at higher Mg^{2+} concentrations. In each case, the peptide synthesis was completely quenched by addition of an excess amount of EDTA, indicating that a slight amount of Mg^{2+} is needed in the systems, probably to preserve the conformation of RNAs.

The mitochondrial system provided a special case: it showed no dependency on the Mg^{2+} concentration, but with low yield, although the other three systems showed some degree of Mg^{2+}-dependency (Fig. 3). In attempting to enhance the yield we found that Mn^{2+} was the most effective enhancer for the peptide synthesis of mitochondria, as shown in Fig. 4a. This Mn^{2+}-effect was not observed in the other three systems (data not shown).

We also investigated the effect of monovalent cation in the peptide synthesis and found that Na^+ and Li^+, which inhibit the aqueous translation system as well as the pyridine system of *E. coli* (data not shown), are able to

TABLE I. **Sensitivity towards antibiotics of pyridine-promoted peptide synthesis systems constructed from various organisms.**[a]

	E. coli	*T. thermophilus*	Yeast	Mitochondria
Incubation temperature (°C)	37	65	37	65
Chloramphenicol	58	57	113	74
Cycloheximide	102	99	58	213[b]

[a]Relative value (%) of peptide synthesis activity with the antibiotic as compared with the activity without the antibiotic (defined as 100%). Pre-treatment of ribosomes with chloramphenicol or cycloheximide and polyphenylalanine synthesis with or without each antibiotic were performed as described in "MATERIALS AND METHODS." [b]The yield increased with the increment of cycloheximide concentration in the range of 0 mM (100% activity) to 250 mM (213% activity), which is at present not interpretable.

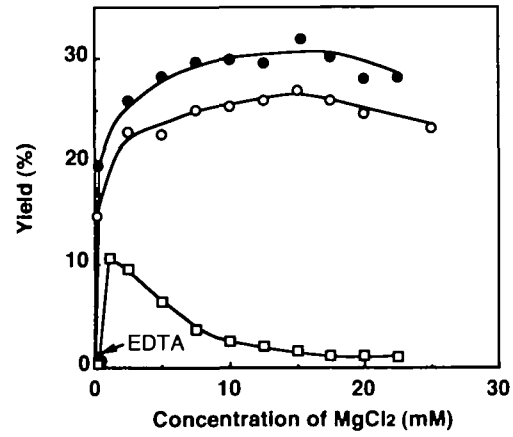

Fig. 3. **Dependency of the polyphenylalanine synthesis on the $MgCl_2$ concentration in the systems of *E. coli* (○), *T. thermophilus* (●), and yeast (□).** "EDTA" indicates complete chelation of free Mg^{2+} in the reaction mixture by addition of excess EDTA.

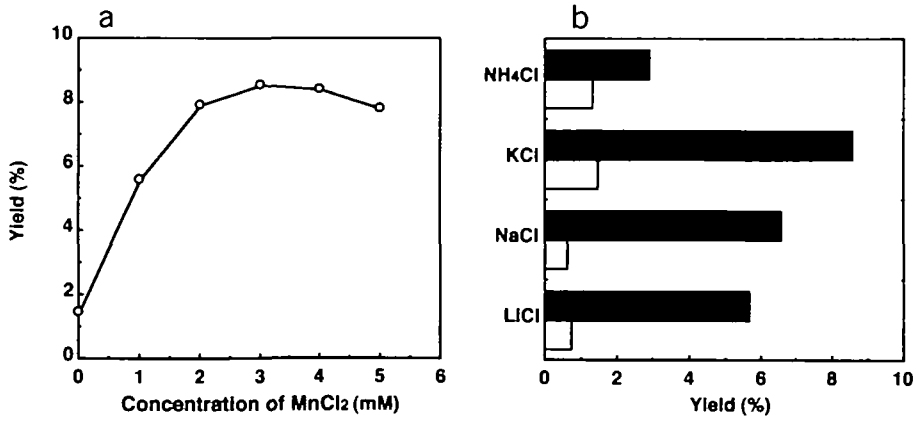

Fig. 4. **Effect of $MnCl_2$ on the polyphenylalanine synthesis in the mitochondrial system.** (a) Dependency of the mitochondrial polyphenylalanine synthesis on $MnCl_2$ concentration. (b) Yield of the mitochondrial polyphenylalanine synthesis with only 15 mM $MgCl_2$ (□) or with 4 mM $MgCl_2$ plus 3 mM $MnCl_2$ (■) in the presence of 120 mM monovalent cations, Li^+, Na^+, K^+, or NH_4^+ as indicated.

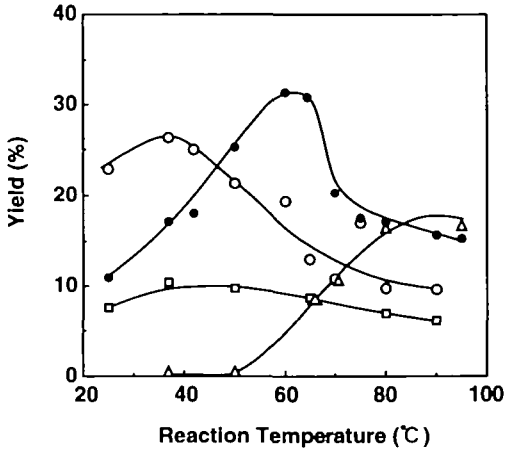

Fig. 5. **Dependency of the polyphenylalanine synthesis on the reaction temperatures in the systems of *E. coli* (○), *T. thermophilus* (●), yeast (□), and bovine mitochondria (△).**

replace K^+ or NH_4^+ efficiently in the mitochondrial system (Fig. 4b).

Effect of the Reaction Temperature in the Peptide Synthesis—The optimum reaction temperatures for the *E. coli*, *T. thermophilus*, and yeast systems were nearly equal to their growth temperatures, *i.e.*, 37°C for *E. coli*, and yeast, and 60°C for *T. thermophilus*. However, in the mitochondrial system, the activity started to increase from 50°C and reached its optimum between 80 and 95°C as shown in Fig. 5. This unique feature suggests that the peptide synthesis may be performed mostly by ribosomal RNA instead of the whole ribosomes in the mitochondrial system, although other possibilities also have to be taken into consideration.

DISCUSSION

In the present study we have shown that not only *E. coli* and *T. thermophilus* ribosomes, but also yeast and mitochondrial ribosomes can produce oligophenylalanines in the presence of 50% pyridine in the *in vitro* translation system without soluble protein factors or energy sources (Figs. 1 and 2) (*4, 5*). The material of the uppermost spot defined in Fig. 2 was already found to contain oligophenylalanines from tetramer up to decamer as analyzed by HPLC (*5*). Larger oligomers, if any, are impossible to analyze because of their insolubility.

The systems prepared from *E. coli*, *T. thermophilus*, and mitochondria were sensitive toward chloramphenicol, whereas that from yeast was sensitive toward cycloheximide, as shown in Table I. These results are consistent with those of the usual aqueous translation systems, thus indicating that the active conformation of the catalytic sites including the peptidyltransferase center are preserved even in the presence of a high concentration of pyridine.

Although chloramphenicol and cycloheximide are scarcely soluble in water (the solubility in water at room temperature is 7.7 and 75 mM, respectively), they are well soluble in pyridine (the solubility is not less than 500 mM). Therefore, the actual concentration of the antibiotics around the active site on ribosomes is much lower in the 50% pyridine system than in the fully aqueous system, if their total concentrations are identical between the two systems. By taking this fact into consideration, a relatively high concentration of antibiotics was used in the pyridine system (2.5 mM chloramphenicol and 25 mM cycloheximide), although these antibiotics are used in the concentration of micromolar order in the usual aqueous translation system. Whereas the inhibition extents by these antibiotics in the aqueous systems were 60–80% (*9*), those in the pyridine systems were as much as 30–40%, probably because of the solubilities of these antibiotics.

All the pyridine systems required Mg^{2+} for the activity, probably because it is necessary for preserving an active conformation of ribosomes. It is unclear why the mitochondrial system requires Mn^{2+}. This may be related to the fact that mitochondria accumulate Mn^{2+} in living cells (*11*), although there has been no report indicating the participation of Mn^{2+} in the translation process.

The mitochondrial system prefers Li^+ as a monovalent cation (Fig. 4b), although a high concentration of Li^+ is known to remove ribosomal proteins from ribosomes, and also prefers very high temperature for the efficient reaction (Fig. 5), although such high temperatures should certainly denature almost all the ribosomal proteins. These facts suggest the possibility that in the mitochondrial system the ribosomal RNAs rather than ribosomal proteins are mainly involved in the peptide synthesis. This speculation requires further experimental evidence.

We thank Miss Chie Takemoto for kindly providing us with bovine mitochondrial ribosomes.

REFERENCES

1. Nomura, M. (1990) History of ribosome research: A personal account in *The Ribosome—Structure, Function & Evolution* (Hill, W.E. *et al.*, eds.) pp. 3-55, American Society for Microbiology, Washington, DC
2. Noller, H.F., Hoffarth, V., and Zimniak, L. (1992) Unusual resistance of peptidyl transferase to protein extraction procedures. *Science* **256**, 1416-1419
3. Schulze, H. and Nierhaus, K.H. (1982) Minimal set of ribosomal components for reconstitution of the peptidyltransferase activity. *EMBO J.* **1**, 609-613
4. Nitta, I., Ueda, T., and Watanabe, K. (1994) Template-dependent peptide formation on ribosomes catalyzed by pyridine. *J. Biochem.* **115**, 803-807
5. Nitta, I., Ueda, T., Nojima, T., and Watanabe, K. (1995) Template-dependent polypeptide synthesis in a factor- and energy-free translation system promoted by pyridine. *J. Biochem.* **118**, 841-849
6. Speeding, G. (1990) Isolation and analysis of ribosomes from prokaryotes, eukaryotes, and organelles in *Ribosomes and Protein Synthesis* (Speeding, G., ed.) pp. 1-29, IRL Press, Oxford
7. Matthews, D.E., Hesseles, R.A., Denslow, N.D., Edwards, J.S., and O'Brien, T.W. (1982) Protein composition of the bovine mitochondrial ribosome. *J. Biol. Chem.* **257**, 8788-8794
8. Freeman, K.B. (1969) Inhibition of mitochondrial and bacterial protein synthesis by chloramphenicol. *Can. J. Biochem.* **48**, 479-485
9. Kuntzel, H. (1969) Proteins of mitochondrial and cytoplasmic ribosomes from *Neurospora crassa*. *Nature* **222**, 142-146
10. Tuite, M.F. (1986) mRNA-dependent yeast cell-free translation systems: Theory and practice. *Yeast* **2**, 35-52
11. Gavin, C.E., Gunter, K.K., and Gunter, T.E. (1992) Mn^{2+} sequenstration by mitochondria and inhibition of oxidative phosphorylation. *Toxicol. Appl. Pharmacol.* **115**, 1-5