〔Chiba Med. J. 52, 243～249, 1976〕

〔原著〕

Synthesis of Intra- and Extracellular Enzymes by Free and Membrane-bound Polyribosomes of *Pseudomonas fluorescens* var. *cellulosa*

AKIRA FUSE* and HIROSHI SUZUKI**

(*Received for Publication, September 30, 1976*)

SUMMARY

Extracellular enzymes, such as cellulase and amylase, were found predominantly in the membrane-bound polyribosome fraction from *Pseudomonas fluorescens* var. *cellulosa*, whereas cellobiase and β-glucosidase known as intracellular enzymes were found almost equally in both the free and membrane-bound polyribosome fraction.

Cellulase and amylase were synthesized also *in vitro* by membrane-bound polyribosomes predominantly, while cellobiase and β-glucosidase by both types of polyribosomes at nearly equal rates. These results suggest that there are distinctive membrane-bound polyribosomes which form the secretory enzymes in prokaryotic cells as in the case of eukaryotic cells, and that polyribosomes are indistinctive which form the intracellular hydrolytic enzymes.

Keywords: Enzyme synthesis, Polyribosome, Extracellular enzyme, Cellulase, Amylase

INTRODUCTION

In bacterial[1], plant[2] and animal[3] cells, polyribosomes exist both in association with membranes and free in the cytoplasm. A characteristic function has been suggested for each of these populations of polyribosomes[4~6]. Indeed, it is generally accepted that in mammalian secretory cells membrane-bound polyribosomes synthesize secretory products, whereas free polyribosomes synthesize proteins which remain in the cells[7~12].

The mechanism of secretion of extracellular enzymes in prokaryotic cells, which do not possess developed secretory apparatus as in eukaryotes has been studied with much interest, but little is known. Recently, *in vivo* formation of amylase by the membrane fraction of *Bacillus amyloliquefaciens*[13,14] and alkaline phosphatase by the membrane-associated polyribosomes of *Escherichia coli*[15] was reported, both the enzymes being proved to be extracellular. However, there are no reports which dealt with the possible relation between the two types of polyribosomes and the synthesis of exportable or non-exportable proteins in a single species of microorganism.

The localization of some intra- and extracellular enzymes in *Pseudomonas fluorescens* var. *cellulosa* has been already demonstrated[16]. The present report deals with the localization and formation of two groups of enzymes in the free and membrane-bound polyribosome fractions prepared from this bacterium.

MATERIALS AND METHODS

Culture conditions—The same strain of *Pseudomonas*

* Department of Microbiology, School of Medicine, Chiba University, Chiba 280.

** Department of Biological Sciences, University of Tsukuba, Ibaragi 300-31.

布施晃，鈴木　怒：*Pseudomonas fluorescens* var. *cellulosa* の膜結合型および遊離型ポリソームによる菌体内および菌体外酵素の合成

千葉大学医学部微生物学教室，筑波大学生物科学系

昭和51年9月30日受付

fluorescens var. *cellulosa* as in a previous paper[17] was grown in a 500-m*l* flask containing 400 m*l* of the basal medium with 1 % (w/v) carboxymethyl cellulose (CM-cellulose) or 0.5 % glucose as a carbon source at 37°C on rotary shaker. The extent of bacterial growth was estimated by measurment of turbidity at 610 nm.

Preparation of free and membrane-bound polyribosomes—Cells harvested at a late logarithmic phase of the culture were fractionated principally according to the method of Carpenter and Barnett[18]. The washed cells were suspended in 0.02 M Tris-HCl buffer, pH 7.8 (T-buffer) containing 10 mM EDTA. At this step the suspension was made up to one-twentieth the volume of original culture. After standing at room temperature for 10 min, the cells were spun down and resuspended in T-buffer containing 0.32 M sucrose and lysozyme (2 mg/m*l*). The mixture was shaken gently at 30°C for 30 min, and the resulting spheroplasts were separated by centrifugation at 10,000×*g* for 20 min. They were washed twice with T-buffer containing 0.32 M sucrose and frozen in liquid nitrogen. The frozen spheroplasts were thawed and suspended in T-buffer containing 10 mM $MgCl_2$ and 70 mM KCl (TMK-buffer). This suspension was homogenized with one stroke of a Teflon homogenizer and centrifuged twice at 14,000×*g* for 20 min to remove the intact cells and unbroken spheroplasts. The supernatant fluid was centrifuged at 30,000×*g* for 20 min to separate the membranes and cytoplasmic fractions. The cytoplasmic fraction was recentrifuged, and free polyribosomes were obtained from the resulting supernatant by centrifugation at 105,000×*g* for 2 hr. The membranes, after washing with TMK-buffer, were treated with 0.5 % DOC (sodium deoxycholate) in TMK-buffer for 40 min and centrifuged at 30,000×*g* for 20 min. From the supernatant from the DOC-treated membrane preparation, polyribosomes previously bound to the membrane were collected by centrifugation at 105,000×*g* for 2 hr. These polyribosome preparations were washed once with TMK-buffer by centrifugation at 105,000×*g* for 2 hr and used for either the determination of enzyme activities preformed *in vivo* or the enzyme synthesis *in vitro*. All operations outlined above were carried out at 0-4°C unless otherwise stated.

Preparation of S-100 fraction—The S-100 fraction was prepared by the method of Nirenberg and Mathaei[19] from glucose-grown cells, which had been proved to contain little or only negligible activities of the enzyme concerned[17].

Determination of enzyme activities and RNA—Activities of cellulase (CM-cellulase, CM-cellulose liquefaction; Avicelase, Avicel-saccharification), amylase and β-glucosidase (*p*-nitrophenyl-β-glucosidase) were assayed as described previously[17,20] and expressed as each enzyme units: CM-cellulase was determined as the increase in specific fluidity per hr, Avicelase as the increase in specific fluidity per hr, Avicelase as the increase in reducing power in terms of absorbancy at 660 nm per 3 days, amylase as the decrease in blue value (after treatment with I_2) per 30 min, and β-glucosidase as the increase in *p*-nitrophenol in terms of absorbancy at 400 nm per 18 hr. Cellobiase activity was assayed by measurment of glucose liberated from cellobiose per 3 hr, using a glucose oxidase-peroxidase system and expressed in terms of absorbancy at 400 nm[21].

Ribonucleic acid was estimated by the orcinol method and absorbancy at 260 nm.

RESULTS

1. Enzyme activities associated with free and membrane-bound polyribosomes.

Cellulase and amylase are typical extracellular enzymes of this bacterium, whereas β-glucosidase and cellobiase localize intracellularly[16]. In order to infer the sites at which these enzymes are formed, activities of the preformed enzymes in two types of polyribosomes were investigated. The results are summerized in Table I. Cellulase activities, assayed as either CM-cellulase or Avicelase, in the membrane-bound polyribosome fraction were apparently higher than

those in the free polyribosome fraction. A similar situation were also found for the activity of amylase. On the contrary, no significant difference was observed in the activities of β-glucosidase or cellobiase between the two types of polyribosome fractions.

Table I. Distribution of enzymes in free and membrane-bound polyribosome fractions. Preparation of free and membrane-bound polyribosomes were treated with 5 mM EDTA to liberate the nascent proteins and the enzyme activities were assayed.

Enzyme	Enzyme activity (units/mg RNA)		Membrane-bound polyribosomes
	Free polyribosomes	Membrane-bound polyribosomes	Free polyribosomes
CM-cellulase	20.8	44.1	2.12
Avicelase	4.9	13.4	2.73
Amylase	21.2	31.4	1.49
Cellobiase	5.8	5.3	0.91
β-Glucosidase	17.7	13.4	0.76

2. Enzyme formation *in vitro* by free and membrane-bound polyribosomes.

Free and membrane-bound polyribosomes were tested for their abilities to incorporate radioactive amino acids into nascent proteins, and to form particular enzymes in a cell-free system using S-100 fraction, which were practically free of these enzymes. Under our conditions where T-buffer containing 70 mM KCl was used, essentially no difference was observed between specific radioactivities of free and membrane-bound polyribosomes. As shown in Fig. 1, free and membrane-bound polyribosomes showed a similar time course of amino acid incorporation and reached a similar maximum level of incorporation during first 10 min, the level being maintained even after 30 min of incubation (not shown in the figure). These observations are consistent with findings by Coleman[22], that the specific activities of free and membrane-bound polyribosomes for protein synthesis were closely similar under the optimal conditions.

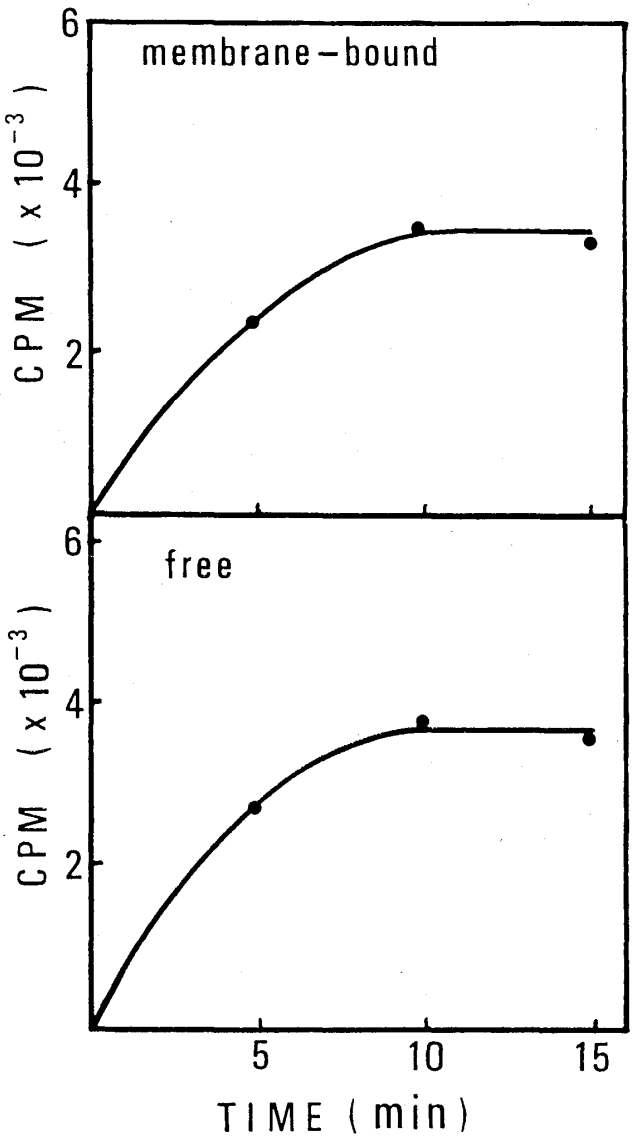

Fig. 1 Time course of [^{14}C]-amino acids incorporation by free and membrane-bound polyribosomes.

[^{14}C]-labelled chlorella protein hydrolysate (0.4 μCi, 40.7 Ci/mmol) was added to the same incubation mixture as for Table 2. At the end of incubation at 30°C, proteins were precipitated by adding 5 m*l* of 5% TCA (trichloroacetic acid) containing 0.5% casein hydrolysate. Precipitates were washed twice by centrifugation and heated in a boiling water bath at 92°C for 30 min. Each suspension was filtered and the radioactivity was measured with a liquid scintillation counter.

In contrast, the enzyme-forming activities of the two polyribosome preparations were quite

Table 2. Enzyme formation by free and membrane-bound polyribosomes. The incubation of polyribosomes for protein synthesis was carried out essentially as described by Nirenberg[19]. The mixture containing, per m*l*, 80 μmoles Tris-HCl buffer at pH 7.8, 8 μmoles $MgCl_2$, 50 μmoles KCl, 6 μmoles β-mercaptoethanol, 2.5 μmoles ATP, 0.5 μmoles GTP, 2.5 μmoles PEP, 10 μg pyruvate kinase, 0.08 μmoles each 20 L-amino acids, 0.5 m*l* polyribosome suspension in S-100 fraction from glucose-grown cells was incubated at 30°C for 20 min. Upon incubating with [^{14}C]-amino acids (0.4 μCi), the radioactivities incorporated were 40,132 and 43,000 cpm/mg RNA for free and membrane-bound polyribosome mixtures, respectively.

Enzyme	Increase in enzyme activity (uits/mg RNA)		Membrane-bound polyribosomes
	Free polyribosomes	Membrane-bound polyribosomes	Free polyribosomes
CM-cellulase	3.96	21.78	5.50
Avicelase	1.53	6.75	4.41
Cellobiase	2.61	2.70	1.03
β-Glucosidase	7.38	3.96	0.54

different (Table 2). The amount of cellulase (CM-cellulase and Avicelase) produced per unit RNA of membrane-bound polyribosomes was more than four times the amount formed by free polyribosomes. Cellobiase was synthesized equally by both types of polyribosomes, and β-glucosidase slightly more by free polyribosomes. Thus the distribution of enzyme-forming abilities in the two types of polyribosomes agreed essentially with that of the enzymes preformed *in vivo* (Table 1).

3. Sucrose density gradient centrifugation analysis.

Sucrose density centrifugation was done with membrane-bound polyribosomes which were incubated with [^{14}C]-amino acids in the system for cell-free protein synthesis (Fig. 2). Both cellulase synthesis and amino acids incorporation were most active in a region where larger polyribosomes should be sedimenting. Thus the data suggested *de nove* synthesis of cellulase in this polyribosome preparation.

DISCUSSION

The results in this paper indicated that the

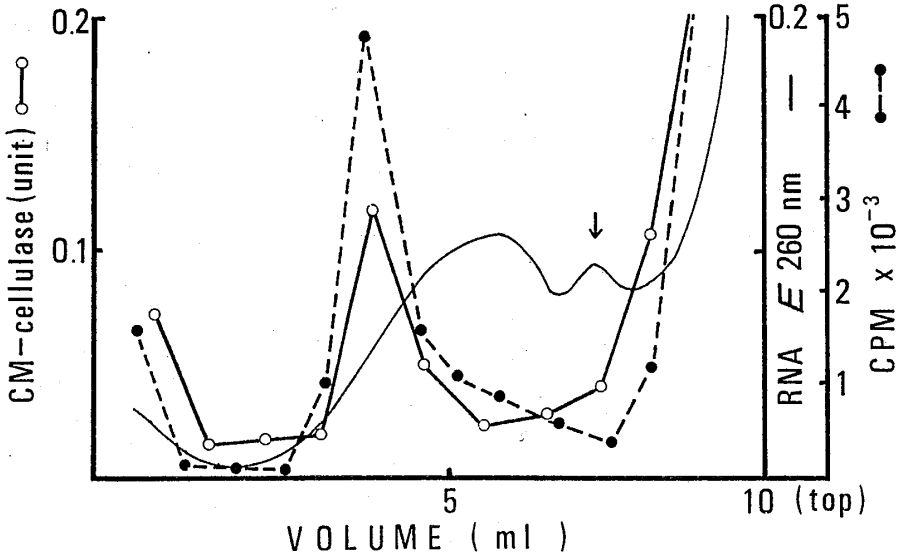

Fig. 2 Sucrose density gradient pattern of membrane-bound polyribosomes incubated with cell-free protein synthesizing system.

The polyribosome fraction incubated in the same incubation mixture as for Fig. 1 at 30°C for 5 min was layered on a 15 to 30% continuous sucrose density gradient containing TMK-buffer and centrifuged at 105,000×*g* for 2 hr. The arrow indicates position of monoribosome (70 S).

extracellular enzymes, cellulase and amylase, are synthesized predominantly by membrane-bound polyribosomes. Such an inclination has been reported for amylase in *Bacillus amyloliquefaciens*[13] and for alkaline phosphatase in *E. coli*[15]. Amylase produced by a membrane fraction was five times as much as that formed in a soluble fraction, and 70 to 80% of alkaline phosphatase nascent polypeptide chains were detected on membrane-associated polyribosomes. These results suggest at least the existence of distinctive polyribosomes participating in the formation of secretory proteins in bacterial cells as well as in eukaryotic cells.

On the other hand, the present results show that the intracellular enzymes, cellobiase and β-glucosidase, are synthesized equally by free and membrane-bound polyribosomes. Thus, no distinctive polyribosomes seem to occur for the formation of intracellular hydrolytic enzymes. With respect to this fact, there may be several possibilities: 1. Cytoplasmic enzymes are formed both by free and by membrane-bound polyribosomes. 2. There are two types of cytoplasmic enzymes, one being formed by free polyribosomes and the other by membrane-bound polyribosomes. 3. All polyribosomes are naturally associated in some manner with membranes: Those forming secretory enzymes are more tightly bound to the membrane than those forming intracellular enzymes or located in special membrane sites as suggested by Schlesinger[15], and free polyribosomes are rather artifacts during the preparation. Our *Pseudomonas* strain possesses both free and bound forms of β-glucosidase[16]*. This fact would have a relation to the second possibility mentioned above.

There arises a question as to what causes the differential function between free and membrane-bound polyribosomes with respect to discrimination of the messenger RNAs for secretory and cytoplasmic enzymes. Several lines of studies showed that an additional protein is contained in either free[33] or bound[34] ribosomes, and initiation factors of translation discriminate the messenger RNAs[25~29]. These findings will have an important role in further studies of this subject.

ACKNOWLEGEMENT

This work was done at Tokyo Kyoiku University.

ま と め

細菌の単離ポリソームによる菌体外酵素合成の報告はあるが，同一細菌の単離ポリソームによる菌体外・菌体内酵素合成の報告はない。*Pseudomonas fluorescens* var. *cellulosa* のセルラーゼは菌体外，β-グルコシダーゼは菌体内にそれぞれ局在している。本実験では本菌のカルボキシメチルセルロース (CMC) 培養から膜結合型および遊離型ポリソームを，またグルコース培養から合型S-100分画をそれぞれ調製し，ニレンバークの無細胞タンパク合成系を用い，これら酵素の合成と2種のポリソームとの関係について調べた。その結果，セルラーゼ活性は膜結合型分画において2倍，また膜結合型分画による合成系での活性増加で約5倍，遊離型分画のものより高い値を示した。一方菌体内酵素のβ-グルコシダーゼ活性は遊離型分画中，およびそれを用いた合成系での活性増加でいくらか膜結合型分画より高い値を示したが，同じ菌体内酵素であるセロビアーゼは両者間に差異が見られなかった。以上の結果，動物の分泌細胞に見られるように，細菌においても菌体外タンパクの合成に関与する膜結合型ポリソームの存在が示唆されたが，菌体内タンパクの合成に関与する特別のポリソームの存在は本実験では見い出し得なかった。

REFERENCES

1) Schlessinger, D., Marchesi, V.T. and Kwan, B. C.K.: Binding of ribosomes to cytoplasmic reticulum of *Bacillus megaterium*. J. Bacteriol. 90, 456-466, 1965.

* Unpublished data show the occurrence in this bacterium of at least two kinds of β-glucoside hydrolases differing greatly in their relative activities toward cellobiose and *p*-nitrophenyl-β-glucoside. Their cellular localization is under investigation.

2) Nicolson, M.O. and Flamm, W.G.: Properties and significance of free and bound ribosomes from cultured tobacco cells. Biochim. Biophys. Acta 108, 266–274, 1965.

3) Siekevits, P. and Palade, G.E.: A cytochemical study on the guinea pig. V. *In vitro* incorportion of leucin-1-C^{14} into the chymotrypsinogen of various cell fractions. J. Biophys. Biochem. Cytol. 7, 619–630, 1960.

4) Uenoyama, K. and Ono, T.: Specificities in messenger RNA and ribosomes from free and bound polyribosomes. Biochem. Biophys. Res. Commun. 49, 713–719, 1972.

5) Shafrits, D.A. and Isselbacher, K.J.: Liver protein synthesis: Differences in the properties of membrane-bound and free ribosomes. Biochem. Biophys. Res. Commun. 46, 1721–1727.

6) Murty, C.N. and Sidransky, H.: Studies on the turnover of mRNA in free and membrane-bound polyribosomes in rat liver. Biochim. Biophys Acta 281, 69–78, 1972.

7) Takagi, M. and Ogata, K.: Direct evidence for albumin biosynthesis by membrane bound polysomes in rat liver. Biochem. Biophys. Res. Commun. 33, 55–60, 1968.

8) Takagi, M., Tanaka, T. and Ogata, K.: Functional differences in protein synthesis between free and bound polysomes of rat liver. Biochim. Biophys. Acta 217, 148–158, 1970.

9) Hicks, S.J., Drysdale, J.W. and Munro, H.N.: Preferential synthesis of ferritin and albumin by different populations of liver polysomes. Science 164, 584–585, 1969.

10) Ganoza, M.C. and Williams, C.A.: *In vitro* synthesis of different categories of specific protein by membrane-bound and free ribosomes. Proc. Natl. Acad. Sci. USA 63, 1370–1376, 1969.

11) Gilbert, J.M.: Translation of messenger RNA fractions extracted from free and membrane-bound rat forebrain ribosomes in a rabbit reticulocyte cell-free system. Biochem. Biophys. Res. Commun. 52, 79–87, 1973.

12) Shafritz, D.A.: Protein synthesis with messenger ribonucleic acid fractions from membrane-bound and free liver polysomes. J. Biol. Chem. 249, 81–88, 1974.

13) Coleman, G.: The distribution of α-amylase-forming ability between the membrane and soluble fractions of cell free preparation of *Bacillus amyloliquefaciens*. Biochem. J. 116, 763–765, 1970.

14) Ninomiya, Y., Imanishi, T., Shinmyo, A. and Enatsu, T.: Immunochemical detection of α-amylase synthesis by protoplast membranes of *Bacillus amyloliquefaciens*. J. Ferment. Technol. 54, 374–382, 1976.

15) Cancedda, R. and Schlesinger, M.J.: Localization of polyribosomes containing alkaline phosphatase nascent polypeptides on membranes of *Escherichia coli*. J. Bacteriol. 117, 290–301, 1974.

16) Yamane, K., Yoshikawa, T., Suzuki, H. and Nisizawa, K.: Localization of cellulase components in *Pseudomonas fluorescens* var. *cellulosa*. J. Biochem. (Tokyo) 69, 771–780, 1971.

17) Yamane, K., Suzuki, H., Hirotani, M., Ozawa, H. and Nisizawa, K.: Effect of nature and supply of carbon sources on cellulase formation in *Pseudomonas fluorescens* var. *cellulosa*. J. Biochem. (Tokyo) 67, 9–18, 1970.

18) Carpenter, S.A. and Barnett, L.B.: Location of cellulase activity in *cellvibrio gilvus*. Arch. Biochem. Biophys. 122, 1–7, 1967.

19) Nirenberg M.W. and Matthaei, J.H.: Characteristics and stabilization of DNAase sensitive protein synthesis in *E. coli* extracts. Proc. Natl. Sci. Acad. USA. 47, 1580–1588, 1961.

20) Yamane, K., Suzuki, H. and Nisizawa, K.: Purification and properties of extracellular and cell-bound cellulase components of *Pseudomonas fluorescens* var. *cellulosa*. J. Biochem. 67, 19–35, 1970.

21) Papadopoulas, N.M. and Hess, W.C.: Determination of neuraminic (sialic) acid, glucose and fructose in spinal fluid. Arch. Biochem. Biophys. 88, 167–171, 1960.

22) Coleman, G.: Comparison of the abilities of

free and membrane-bound polyribosomes isolated from exponential-phase cells of *Bacillus amyloliquefaciens* to incorporate amino acids into protein. Biochem. J. 115, 863–864, 1969.

23) Borgese, G., Blobel, G. and Sabatini D.D.: *In vitro* exchange of ribosomal subunits between free and membrane-bound ribosomes. J. Mol. Biol. 74, 415–438, 1973.

24) Scheinbuks, J., Kaltschmidt, E. and Marcus, L.: The presence of an additional protein associated with membrane-bound ribosomes of *Azotobacter vinelandii*. Biochim. Biophys. Acta 281, 141–144, 1972.

25) Yoshida, M. and Rudland, P.S.: Ribosomal binding of bacteriophage RNA with different components of initiation factor F3. J. Mol. Biol. 68, 465–481, 1972.

26) Grover, Y., Pollack, Y., Berissi, H. and Revel, M.: Cistron specific translation control protein in *Escherichia coli*. Nature New Biology 239, 16–18, 1972.

27) Wigle, D.T.: Purifiation of a messenger-specific initiation factor from ascites-cell supernatant. Eur. J. Biochem. 35, 11–17, 1973.

28) Leffler, S. and Szer, W.: Messenger selection by bacterial ribosomes. Proc. Natl. Acad. Sci. USA. 70, 2364–2368, 1973.

29) Steitz, J.A.: Discriminatory ribosome rebinding of isolated regions of protein synthesis initiation from the ribonucleic acid of bacteriophage R17. Proc. Natl. Acad. Sci. USA. 70, 2605–2609, 1973.