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Requirement of a Large Amount of Polyuridylate for Efficient Polyphenylalanine Synthesis by Staphylococcal Ribosomes

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Polyuridylic-acid-directed polyphenylalanine [poly(U)-directed poly(Phe)] synthesis by *Staphylococcus aureus* ribosomes (washed in 1.0 M NH_4Cl) increased linearly with increasing amount of poly(U): the synthesis by washed staphylococcal ribosomes in the presence of about 800 μg poly(U)/ml reached the same level as that by ribosomes of *Escherichia coli* Q13 at the optimum poly(U) concentration (about 40 μg/ml). Binding of either 70S ribosomes or 30S ribosomal subunits of *S. aureus* U9 to poly(U) was less than that of *E. coli* Q13. Sedimentation analysis in a sucrose gradient showed that the amount of the heavy complex produced by interaction of poly(U) and ribosomes of *S. aureus* U9 was small at a high poly(U) concentration (1750 μg/ml), whereas that of *E. coli* Q13 was large even at 500 μg poly(U)/ml. These results suggest that low ability of the washed *S. aureus* ribosomes to synthesize poly(Phe) chiefly results from poor affinity of ribosomes for poly(U), since no ribonuclease activity could be found in the washed ribosomes.

Keywords——ribosome; *S. aureus*; *E. coli*; polyphenylalanine synthesis; protein synthesis

There are a number of antibiotics which inhibit protein synthesis in Gram-positive bacteria. Nevertheless, most studies on the molecular basis of the antibiotic action have been conducted in a cell-free system of *Escherichia coli* because the incorporation of amino acids into protein by cell-free extracts of Gram-positive bacteria is relatively poor.[1,2] Mao[3] has succeeded in realizing active protein synthesis by cell-free extracts of *Staphylococcus aureus*. He discussed major differences in the characteristics of cell-free extracts of *S. aureus* and *E. coli* from the viewpoint of magnesium concentration requirements for maximal protein synthesis. In this paper, with the aim of obtaining efficient polyphenylalanine [poly(Phe)] synthesis, we compared the nature of the synthesis by cell-free extracts of *S. aureus* and *E. coli* in preliminary experiments.

Materials and Methods

Bacteria and Preparation of Cell Fraction——*S. aureus* U9[4] was used as a representative strain of Gram-positive bacteria and *E. coli* Q13 as a Gram-negative bacterium. Cell fractions were prepared according to the method of Mao.[3]

Polyuridylic-Acid [Poly(U)]-Directed Poly(Phe) Synthesis——The reaction mixture (0.2 ml) of 10 mM potassium-*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonate (HEPES) buffer (pH 7.6), 16 mM magnesium acetate ($MgAc_2$), 50 mM NH_4Cl, 0.1 mM dithiothreitol, 1.0 mM adenosine triphosphate, 0.05 mM guanosine triphosphate, 5 mM phosphoenolpyruvate, 8 μg of pyruvate kinase, 2 A_{260} units of ribosomes, 200 μg of S-100 fraction (S-100: 105000 × *g* supernatant), 0.1 μCi of ^{14}C-phenylalanine (specific activity: 522 mCi/mmol) and various amounts of poly(U) was incubated for 30 min at 37 °C, and the incorporation of ^{14}C-phenylalanine into hot trichloroacetic acid-precipitable material was assayed by a filter paper disk method.[5] In the experiments, unless otherwise indicated, the washed ribosomes used were prepared by washing in 10 mM potassium-HEPES buffer (pH 7.6) containing 1.0 M NH_4Cl, 16 mM $MgAc_2$ and 0.1 mM dithiothreitol.

Poly(U) Binding to Ribosomes——About one A_{260} unit of either 70S ribosomes or 30S ribosomal subunits and various amounts of ^{3}H-poly(U) (specific activity: 596 cpm/mg) were mixed in a total volume of 1.0 ml of the binding buffer, *i.e.* 10 mM Tris-acetate buffer (pH 7.6) containing 50 mM NH_4Cl and 20 mM $MgAc_2$, at 0 °C. After a 5 min incubation, the reaction mixture was filtered through an alkali-treated nitrocellulose membrane as described by Smolasky and Tal.[6]

Sedimentation Analysis for Complex of Poly(U) and Ribosomes——About 30 A_{260} units of ribosomes and various amounts of poly(U) were mixed in a total volume of 1.0 ml of Tris-acetate buffer (pH 7.6) containing 16 mM $MgAc_2$, 50 mM NH_4Cl and 0.1 mM dithiothreitol, and incubated at 20 °C for 10 min. The mixture was layered on 15 ml of a 5—20% (w/v) sucrose density gradient in the above medium (except that the $MgAc_2$ concentration was 0.3 mM) and centrifuged at 52000 × *g* for 16 h at 2 °C in the RPS-25-3A rotor of a Hitachi 65P centrifuge. Thirteen-drop fractions were collected from the bottom of the tube and the absorbance of each fraction at 260_{nm} was measured.

Results and Discussion

The effects of transfer ribonucleic acid (tRNA), S-100, ribosomes and poly(U) on the poly(U)-directed poly(Phe) synthesis in *S. aureus* U9 were examined. Poly(Phe) synthesis by cell-free extracts of *S. aureus* U9 increased by increasing the amount of poly(U) (Fig. 1) was unaffected by increased amounts of tRNA and S-100, but was markedly decreased by a large amount of ribosomes (data not shown). The last observation may be due to the decrease in the amount of poly(U) per ribosome, as inferred later from various results. As a first attempt to investigate which extract, either S-100 or ribosomes from staphylococcal cells, is involved in the poor poly(Phe)-synthesizing ability, we tested poly(Phe)-synthesizing ability in terms of four possible, *i.e.* two homologous and two heterologous, combinations of ribosomes and S-100 which were prepared from *S. aureus* U9 and *E. coli* Q13 (Fig. 1). When the cell-free extracts contained ribosomes of *S. aureus* U9 (U9 ribosomes), poly(Phe) synthesis increased steadily and smoothly with increasing amount of poly(U), whereas the synthesis by ribosomes of *E. coli* Q13 (Q13 ribosomes) increased sharply up to the maximum at 40 μg poly(U)/ml and then gradually decreased from 100 to 900 μg/ml of poly(U) (Fig. 1). This result suggests that the propensity for poor poly(U)-utilization is intrinsic to staphylococcal ribosomes, since the poly(Phe) synthesis by ribosomes of *S. aureus* 209P showed the same activity as that of strain U9 (data not shown). Takanami and Okamoto[7] have reported that a high concentration of poly(U) is not favorable for the formation of polysomes by *E. coli* ribosomes. Their report seems consistent with our results on poly(Phe) synthesis by Q13 ribosomes (Fig. 1).

To determine why U9 ribosomes need such a large amount of poly(U), the possibility of contamination by ribonuclease (RNase) in the ribosomes was first examined, using 2′- or 3′-uridylic acid (UMP) as an end product inhibitor. Poly(Phe) synthesis by U9 ribosomes was

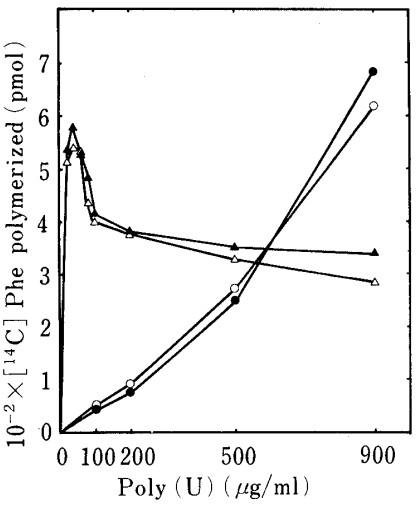

Fig. 1. Effect of Poly(U) Concentration on Poly(Phe) Synthesis by Homologous and Heterologous Systems Composed of Washed Ribosomes and S-100 of either *S. aureus* U9 or *E. coli* Q13

The radioactivity in the absence of poly(U) (background) was equivalent to 8.7 pmol/mg of ribosomal protein. Symbols: (●), homologous system containing U9 ribosomes and U9 S-100; (○), heterologous system containing U9 ribosomes and Q13 S-100; (▲), heterologous system containing Q13 ribosomes and U9 S-100; (△), homologous system containing Q13 ribosomes and Q13 S-100.

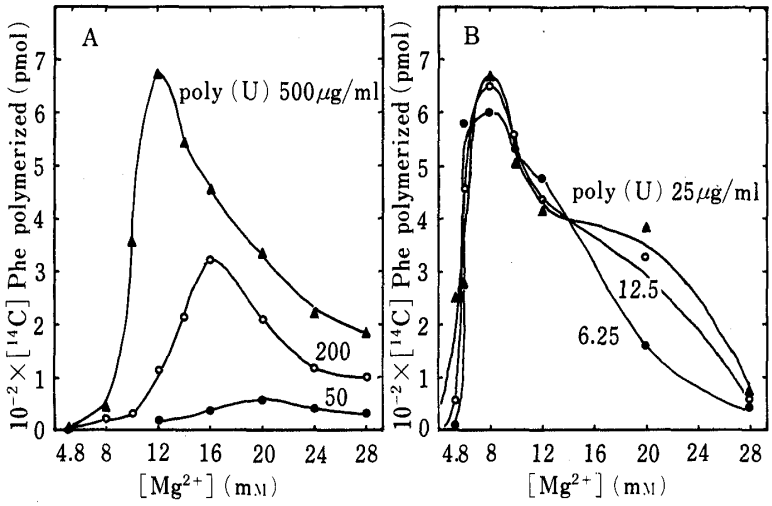

Fig. 2. Effect of Mg^{2+} Concentration in the Presence of Various Amounts of Poly(U) on Poly(U)-Directed Poly(Phe) Synthesis by Washed Ribosomes of *S. aureus* U9 (A) and *E. coli* Q13 (B)

Background was equivalent to 13.8 pmol/mg of ribosomal protein.
Symbols: (●), (○) and (▲) represent 50, 200 and 500 μg poly(U)/ml in (A) and 6.25, 12.5 and 25 μg poly(U)/ml in (B), respectively.

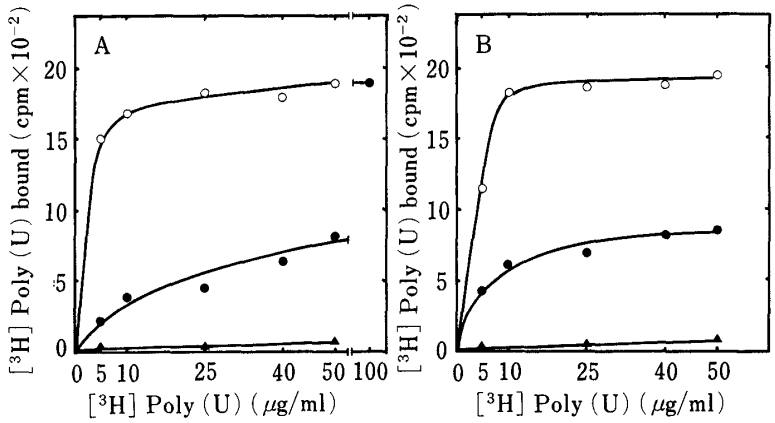

Fig. 3. Binding of ^3H-Poly(U) as a Function of Poly(U) Concentration to 70S Ribosomes (A) and 30S Ribosomal Subunits (B) from *S. aureus* U9 and *E. coli* Q13

Symbols: (●), 70S ribosomes or 30S ribosomal subunits of strain U9; (○), 70S ribosomes or 30S ribosomal subunits of strain Q13; (▲), in the absence of ribosomes or ribosomal subunits.

not affected by either 2′-UMP or 3′-UMP. Then, the polynucleotide and staphylococcal ribosomes were incubated at 37 °C for 10 min in a reaction mixture containing 8 mM $MgAc_2$ and 10 μg of poly(U). The remaining activity of poly(U) was determined by addition of S-100 and Q13 ribosomes to the resultant mixture. There was no loss of poly(U) (data not shown). Thus, the possibility of RNase contamination in the staphylococcal ribosomes was ruled out.

Mao[3] found that the Mg^{2+} concentration required for maximum incorporation in cell-free protein synthesis of *S. aureus* was much higher (16 to 22 mM) than in *E. coli* (7 to 11 mM). The effect of Mg^{2+} on poly(Phe) synthesis by U9 ribosomes, therefore, was compared with that on Q13 ribosomes in the presence of various amounts of poly(U). In the case of synthesis by U9 ribosomes, the optimal magnesium profile was shifted to lower magnesium concentrations with increase of poly(U) concentration [Fig. 2(A)], whereas that required for

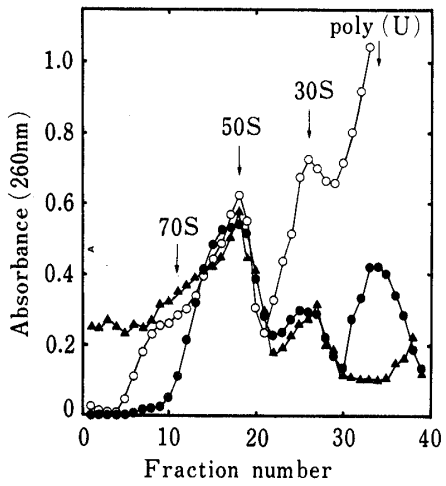

Fig. 4. Sedimentation Analysis of Mixtures of Poly(U) and Washed Ribosomes of *S. aureus* U9 and *E. coli* Q13 in the Presence of 0.3 mM Mg^{2+}

Symbols: (●), 500 μg/ml of poly(U) and U9 ribosomes; (○), 1750 μg/ml of poly(U) and U9 ribosomes; (▲), 500 μg/ml of poly(U) and Q13 ribosomes.

polypeptide synthesis by Q13 ribosomes was unchanged (8 mM $MgAc_2$) [Fig. 2(B)]. The molecular basis of this observation is unclear at present.

Second, the binding ability of ribosomes of *S. aureus* and *E. coli* for poly(U) was determined[6] by the method using an alkali-treated nitrocellulose filter. The amounts of poly(U) bound to 70S ribosomes and 30S ribosomal subunits of *S. aureus* U9 were far less than those in the case of *E. coli* Q13, and only reached the same level as that of *E. coli* Q13 at 100 μg poly(U)/ml [Fig. 3(A)]. Thus, the poly(U)-binding ability of ribosomes was determined at high concentrations of poly(U) by sedimentation analysis in a sucrose gradient. U9 ribosomes formed only a small amount of heavy complex at 1750 μg poly(U)/ml, while at 500 μg poly(U)/ml formed no heavy complex corresponding to fractions of less than No. 10 in Fig. 4, whereas Q13 ribosomes produced fair amounts of heavy complexes, *i.e.* polysomes (Fig. 4).

The findings suggest that the low ability of *S. aureus* cell-free extracts to synthesize poly(Phe) chiefly results from poor affinity of ribosomes for poly(U)—such an affinity may reflect characteristics of the 30S ribosomal subunits of the bacteria—and that the ability, in extracts that contain washed ribosomes and the optimum amount of $MgAc_2$ required to obtain the maximal poly(Phe) synthesis, can markedly be improved in the presence of a large amount of poly(U). This distinct poly(Phe)-synthesis dependent on a large amount of poly(U), even if it does not accurately reflect *in vivo* protein synthesis, should provide a means of investigating protein synthesis inhibitors in Gram-positive bacteria, since in our preliminary test, natural messenger ribonucleic acid (mRNA) such as MS2 phage RNA concentrations (220 μg/ml) required for maximum incorporation of ^{14}C-amino acid mixture into protein by *S. aureus* extracts were also about three times as much as those in the case of *E. coli* Q13 extracts (80 μg/ml) at the optimal Mg^{2+} concentrations (11.3 and 8 mM, respectively).

Ribosomal protein S1 of *E. coli* is known to stimulate the binding of poly(U) to ribosomes.[8-12] Since washed ribosomes were used in our present experiments, the possibility cannot be excluded that the poor affinity of staphylococcal ribosomes for poly(U) was due to easier removal of S1-like protein from *S. aureus* than from *E. coli* ribosomes. In fact, when poly(U) (125 μg/ml) was mixed at 0 °C with unwashed U9 ribosomes (5 A_{260} units), the amount of ^3H-poly(U) bound to the unwashed ribosomes was about five times as much as that to U9 washed ribosomes, and this amount was approximately equal to that of washed ribosomes of *E. coli* Q13 (data not shown). On the other hand, the activity of poly(Phe) synthesis by U9 unwashed ribosomes was about half that by washed ribosomes of the same strain (although no RNase activity could be detected in the unwashed ribosomes, when RNase activity based upon liberation of acid-soluble oligonucleotides was measured by the

method of Kalnitsky *et al.*[13]). Further studies into these contradictory results are in progress.

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