A highly efficient poly(U)-dependent poly(Phe) synthesis system for the extreme halophile archaebacterium *Halobacterium halobium*

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A poly(Phe) synthesis system was optimized for the archaebacterium *Halobacterium halobium*. It is essential for maximal activity to isolate the ribosomes from cells in mid-log phase. The system is characterized by the presence of tRNA from brewer's yeast, 6.4 M monovalent cations, 60 mM magnesium, 2 M sulphate anions, a pH of 7.4, and an incubation temperature of 40°C; polycations such as spermidine are not required. Under these conditions one *H. halobium* ribosome synthesizes statistically more than 20 Phe per h, and the synthesis is not exhausted after 2 h (40 Phe/ribosome). The yield of poly(Phe) is one to two orders of magnitude larger than corresponding yields described for other archaebacterial ribosomes. The accuracy of tRNA selection was determined, and an error fraction of about 0.5% was found. More than 30% of the ribosomes participate in poly(Phe) synthesis.

Archaebacterial ribosome *Poly(Phe) synthesis* *Ribosomal accuracy*

1. INTRODUCTION

Our analysis of the tRNA-binding capacity of *E. coli* ribosomes [1] has led to a 3-site model for the elongation cycle [2]. To test whether this 3-site model (valid for *E. coli* ribosomes) is a general description of the ribosomal elongation cycle, we decided to analyse tRNA binding and translocation in ribosomes of a different kingdom, the archaebacteria.

A prerequisite of such an analysis is an efficient protein synthesizing system, in which a significant fraction of ribosomes participates. In *E. coli* systems, a ribosome statistically polymerizes 20–100 Phe, whereas with archaebacterial ribosomes only systems of very low efficiency have been described, e.g., 0.15–0.3 Phe/ribosome per h in the case of the extreme halophile *Halobacterium* [3,4], and 2–6 Phe/ribosome per h in systems with other archaebacterial species [5–7]. Here, we describe an in vitro system for *Halobacterium halobium* with a synthesis rate of 20–35 Phe/ribosome per h and an error frequency of only 0.5%.

2. MATERIALS AND METHODS

2.1. *Materials*

L-[U-^{14}C]Phenylalanine (18.6 GBq/mmol equivalent to 504 mCi/mmol), L-[2,6-^{3}H]phenylalanine (1.81 TBq/mmol equivalent to 49 Ci/mmol) and L-[4,5-^{3}H]leucine (5.37 TBq/mmol equivalent to 145 Ci/mmol) were purchased from Amersham. Poly(U), bulk tRNA and tRNA^{Phe} derived from brewer's yeast were obtained from Boehringer Mannheim.

2.2. *Organism and growth conditions*

H. halobium S9, kindly provided by Dr N. Kamo (Sapporo, Japan) was used. The growth

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medium contained 0.75% casamino acids (Difco), 1% yeast extract (Difco), 10 mM trisodium citrate, 27 mM potassium chloride, 4.3 M sodium chloride, and 75 mM magnesium sulphate. The salts were added as a solid material, which dissolved completely during sterilization at 120°C for at least 30 min. The pH was adjusted to 7.2 with a sterilized NaOH solution (50% w/v), about 100 ml per 100 l medium.

Cells were cultivated at 37°C under continuous aeration in 100 l medium in the presence of antifoam (Wacker Silicone SLE, Wacker-Chemie, Munich; a 10-times diluted solution was sterilized, 10–15 ml of which was added per 12 h) and harvested after 1.5 days at mid-log phase (0.15 A_{560} units per ml), the generation time during log phase being 5.6–6 h. About 100 g cells per 100 l medium were obtained, and these cells were stored at −80°C.

2.3. *Preparation of ribosomes and S-100 fraction*

All procedures were performed at 0–4°C. Typically, 30–40 g cells were ground for 3 min in a Retsch mill with 60–80 g alumina (Alcoa A-305) and 3–4 ml ribosome buffer (30 mM Tris-HCl, pH 7.6, 3.4 M KCl, 60 mM magnesium acetate, 7 mM 2-mercaptoethanol). Then 60–80 ml of the same buffer was added, and the paste was homogenized for 2 min. The crude extract was obtained by two low-speed centrifugations (30 min at 30000 × *g*), and was then subjected to high-speed centrifugation (17 h at 100000 × *g*). The upper 3/4 of the supernatant was dialyzed for 8 h against supernatant buffer (10 mM Tris-HCl, pH 7.6, 2.2 M $(NH_4)_2SO_4$, 60 mM magnesium acetate, 5% glycerol). After dialysis the $(NH_4)_2SO_4$ concentration was raised to 70% saturation by adding 2.38 g per 10 ml, and after a low-speed centrifugation (30 min at 30000 × *g*) the pellet was resuspended in 2–5 ml of the supernatant buffer, dialyzed overnight against supernatant buffer and stored in small portions as S-100 fraction at −80°C.

The ribosomal pellet from the high-speed centrifugation was resuspended in the ribosome buffer and also stored at −80°C. 1 A_{260} unit of 70 S ribosomes was taken as 24 pmol.

2.4. *Preparation of AcPhe-tRNA^{Phe}*

110 nmol $tRNA^{Phe}$ from brewer's yeast was incubated in a 10 ml reaction mixture containing 100 mM Tris-HCl, pH 7.6, 3 mM ATP, 3 mM GTP, 15 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 500 nmol [^{14}C]Phe (or non-labeled Phe) and 1.6 ml wheat germ S-100 (tRNA free, a kind gift from P. Wurmbach, Berlin) at 30°C for 20 min. Then, 0.5 ml of a sodium acetate solution (20%, w/v, pH 5.4) was added. Phenolization, acetylation and purification of AcPhe-$tRNA^{Phe}$ on BD-columns were as described [8].

2.5. *Poly(U)-dependent poly(Phe) synthesis system*

One assay contained in 100 μl 2 A_{260} units of ribosomes, 100 μg poly(U), 20 μg $tRNA^{Phe}$ from brewer's yeast, an optimal amount of S-100 fraction (5–30 μl), 30 mM Tris-HCl, final pH 7.4, 2.0 M KCl, 0.4 M NH_4Cl, 2.0 M $(NH_4)_2SO_4$, 60 mM magnesium acetate, 2.1 mM ATP, 0.5 mM GTP, 5 mM phospho*enol*pyruvate, 2 μg pyruvate kinase, 7 mM 2-mercaptoethanol, and 100 μM [^{14}C]phenylalanine (13 mCi/mmol). The procedure was as follows:

First, a suitable amount of a poly(U) mixture (60 μl/assay) was prepared containing 50 mM Tris-HCl, pH 9.5, 0.67 M NH_4Cl, 0.27 M $(NH_4)_2SO_4$, 100 mM Mg acetate, 3.5 mM ATP·2 Na, 0.83 mM GTP·2Li, 8.33 mM phospho*enol*pyruvate (neutralized with NaOH), 2 μg pyruvate kinase, 11.7 mM 2-mercaptoethanol, 20 μg $tRNA^{Phe}_{yeast}$, 100 μg poly(U), and 167 μM [^{14}C]phenylalanine (13 mCi/mmol). Then the assay was set up with subsequent additions. First, 14.9 mg KCl and 14.6–23.3 mg $(NH_4)_2SO_4$ (the exact amount depends on the amount of S-100 fraction per assay and was adjusted to give a final concentration of $(NH_4)_2SO_4$ of 2 M) are added to each assay tube. Then the following solutions were added at room temperature in order: 10–20 μl H_2O to adjust the assay volume to 100 μl; 60 μl poly(U) mixture; an optimal amount of S-100 fraction (5–30 μl); after mixing by vortexing the ribosomes were added (2 A_{260} units in 2–5 μl ribosome buffer). Usually, the final solution was turbid and precipitates were observed.

The mixture was incubated at 40°C for 45 min, then a drop of 1% (w/v) bovine serum albumin and 2 ml of 5% trichloroacetic acid were added, and, after heating at 90°C for 15 min, the precipitates were collected on glass-fiber filters (Schleicher & Schüll, 23 mm diameter), washed

twice with 2 ml of 5% trichloroacetic acid and once with ether-ethanol (1 : 1), before determining the radioactivity.

2.6. *Misreading system*

235 pmol 70 S ribosomes in a total volume of 250 μl were incubated with 500 μg poly(U) and, where indicated, a 3-molar excess of non-labeled AcPhe-tRNA over ribosomes, in the presence of 30 mM Tris-HCl, pH 7.4, 2.0 M KCl, 2.0 M $(NH_4)_2SO_4$, 0.4 M NH_4Cl, 60 mM magnesium acetate and 7 mM 2-mercaptoethanol at 40°C for 30 min. Next, 250 μl of a solution with the same ionic condition was added, containing 500 μg bulk tRNA from brewer's yeast, 44 μM [^{14}C]Phe (spec. act. 24 cpm/pmol), 22 μM [^{3}H]Leu (22000 cpm/pmol), 4.2 mM ATP, 1.0 mM GTP, 10 mM phospho*enol*pyruvate and 10 μg pyruvate kinase. Poly(Phe) synthesis at 40°C was started by the addition of 50 μl S-100 fraction. At the times indicated 55 μl containing 23.5 pmol 70 S ribosomes was withdrawn and pipetted into 2 ml of 5% trichloroacetic acid, one drop of bovine serum albumin (1% w/v) was added, the mixed solution was heated (90°C for 15 min), filtered through nitrocellulose filter (Sartorius, Göttingen, FRG, no.11306), and washed twice with 2 ml of 5% trichloroacetic acid. After drying, the filters were burnt in a Packard sample oxidizer, which separates the ^{14}C- and ^{3}H-labelled isotopes to the extent of practically 100%. The resulting cpm values were corrected for background, which was obtained in a control assay (minus ribosomes).

3. RESULTS AND DISCUSSION

First, we investigated whether the growth phase of the cells (log phase, stationary phase, etc.) affects the activity of the isolated ribosomes. Fig.1 shows that the highest activity of poly(U) directed poly(Phe) synthesis was found with ribosomes derived from mid-log phase cells. The ribosomes of semi-log cells were active to the extent of only 20%, whereas a negligible activity of below 1% was found in ribosomes derived from stationary phase cells. Therefore, the harvesting point for the standard isolation procedure was fixed at 0.15 A_{560} units per ml.

In the next series of experiments the various

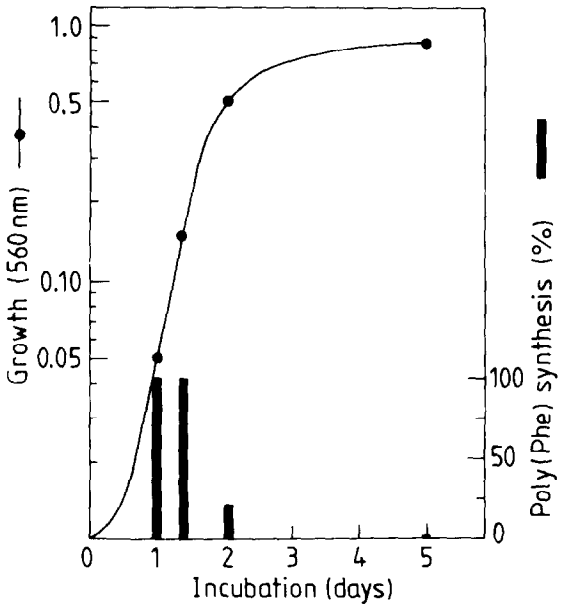

Fig.1. Growth curve of *Halobacterium halobium* (——). Aliquots were withdrawn at the times indicated, the cells harvested and ribosomes isolated. The activities of the ribosomes in poly(Phe) synthesis are indicated as bars at the corresponding growth-curve points.

components needed for poly(Phe) synthesis were systematically optimized starting from a system similar to that described by Bayley and Griffiths [3]. This system did not yield reproducible results in our hands; a variance from 1 to 10 Phe incorporated/70 S ribosome per h was found from one experiment to another for unknown reasons. The concentrations of the relevant components and the incubation parameters are summarized in fig.2A. The optimal values determined were kept constant in the subsequent experiments. For example, in panel B the variable is poly(U), and the optimal amount of poly(U) chosen for the final optimized system is 100 μg/assay and is indicated by an arrow.

As a source of tRNA, we first tried to isolate a tRNA fraction from *H. halobium* but without success. Therefore, $tRNA^{Phe}$ from *E. coli*, baker's yeast, brewer's yeast, and wheat germ was tested. The highest activity was found with brewer's yeast $tRNA^{Phe}$, whereas only half the activity was detected with an equivalent amount of *E. coli* $tRNA^{Phe}$, and no appreciable activity could be found with wheat germ $tRNA^{Phe}$. Fig.2C

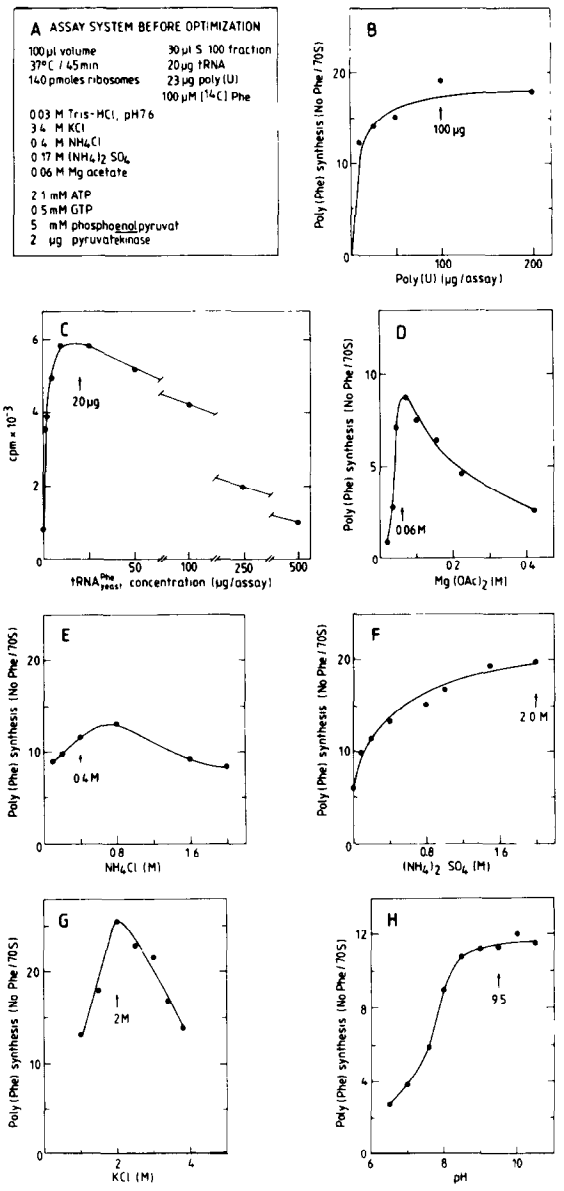

Fig.2. Optimization of the poly(U)-dependent poly(Phe) synthesis system. A, starting condition. B–H, optimization of the components indicated. Whenever an optimum was found (arrow), it was fixed for the subsequent optimization assays. In panel C, 6000 cpm corresponds to 1.3 Phe residues incorporated per 70 S.

demonstrates the optimization for $tRNA^{Phe}$ from brewer's yeast, the chosen optimal value being 20 μg/assay. The curves for magnesium acetate and NH_4Cl (fig.2D and E, respectively) demonstrate that the highest activity was found at

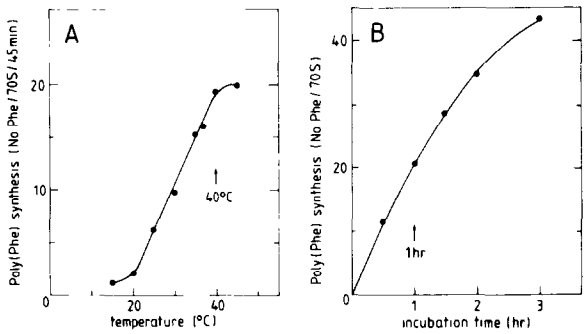

Fig.3. Poly(U)-dependent poly(Phe) synthesis, A, at different temperatures; B, kinetics under optimized condition. The arrows in A and B indicate the values selected for the optimized system.

70 mM Mg^{2+} and 800 mM NH_4Cl, respectively. However, a fine-tuning experiment in the final optimized system revealed that 60 mM Mg^{2+} and 400 mM NH_4Cl yielded optimal activities. With $(NH_4)_2SO_4$ the highest activity was seen at 2 M, but a distinct activity peak was not evident (fig.2F) in contrast to KCl, where a sharp activity peak was found at 2 M (see fig.2G).

Finally, the pH was optimized. Since it is difficult to adjust the pH precisely at extremely high salt concentrations, we varied the pH between 6.5 and 10.5 (abscissa in fig.2H) of the 1.0 M Tris-HCl stock solution used to prepare the poly(U) mixture. We chose 9.5 as the optimal pH for the stock solution of Tris-HCl yielding a pH of 7.4 in the final assay mixture.

Some additional parameters were tested with the optimized system. Addition of increasing amounts of NaCl leads to a continuous decrease of the activity. The addition of polycations such as spermine or spermidine had no significant effect. The optimization of the incubation temperature is depicted in fig.3A, and we chose 40°C as optimal temperature.

The kinetics of poly(Phe) synthesis in the optimized system demonstrates a linear synthesis up to 1 h of incubation (fig.3B), and even after 3 h incubation the system is not exhausted. For standard assays we used an incubation time of 1 h.

Finally, the optimized system was used to determine the accuracy of tRNA selection of the *H. halobium* ribosomes, as well as the percentage of ribosomes taking part in protein synthesis. Fig.4A demonstrates a constant error fraction of about

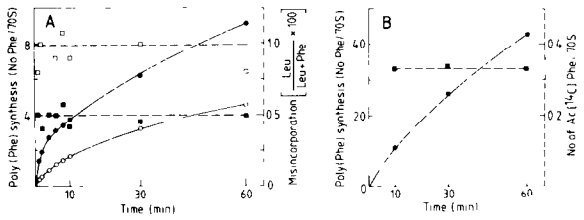

Fig.4. Poly(U)-dependent poly(Phe) synthesis with 70 S ribosomes primed with AcPhe-tRNA. A, misincorporation (– – –) in the course of poly(Phe) synthesis (——) with (●, ■) or without (○, □) prebound AcPhe-tRNA. B, fraction of ribosomes carrying an Ac[^{14}C]Phe-tRNA (– – –) in the course of poly(Phe) synthesis (——).

1% in the course of poly(Phe) synthesis. The relatively low rate of poly(Phe) synthesis in this experiment (9 Phe/70 S per h as compared to 20 Phe/70 S per h in fig.3D) is due to the presence of bulk tRNA which we had to use in the misreading assay. When the ribosomes were primed with AcPhe-tRNA, the synthesis rate was enhanced by a factor of two and the error decreased to 0.5%. This indicates that the initiation event is rate-limiting for poly(Phe) synthesis and is particularly prone to error, in a similar way to that observed for *E. coli* ribosomes [9,10]. When the ribosomes were primed with labeled Ac[^{14}C]Phe-tRNA before they were transferred into the standard poly([^{3}H]Phe) synthesis system an accelerated poly(Phe) synthesis was again observed (40 Phe/70 S per h, see fig.4B). The fraction of ribosomes carrying an Ac[^{14}C]Phe residue remained constant at 0.33. Therefore, at least 33% of the ribosomes took part in poly(Phe) synthesis.

4. CONCLUSIONS

It is important that ribosomes are derived from mid-log phase cells. The system described allows highly efficient and accurate poly(Phe) synthesis, in which a significant fraction of ribosomes participates and one ribosome statistically can polymerize more than 40 phenylalanines per h, i.e., 120 phenylalanines per active ribosome (since 33% of the ribosomes are active in elongation). Therefore, this system is suitable for the analysis of the tRNA binding capacity and the principles of the elongation cycle in *H. halobium* ribosomes.

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