

## Two-dimensional crystalline sheets of *Bacillus stearothermophilus* 50S ribosomal subunits containing a nascent polypeptide chain

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**Summary** — Polylysine chains were synthesized on *Bacillus stearothermophilus* ribosomes in a poly(A)-programmed *in vitro* system. After separation of the ribosomal subunits by sucrose gradient centrifugation, the polylysine chains (in contrast to the polyphenylalanine chains synthesized in a poly(U) system) reproducibly remained attached to the large ribosomal subunit. It was possible to produce two-dimensional crystalline sheets from the large ribosomal subunits containing the polylysine chains. These sheets are an essential prerequisite for three-dimensional reconstruction studies aiming to show that the tunnel in the large ribosomal subunit provides a path for the nascent polypeptide chain.

**ribosomes / *Bacillus stearothermophilus* / crystalline sheets / ribosomal tunnel**

### Introduction

Three-dimensional image reconstruction studies with negatively stained two-dimensional crystalline sheets of 50S ribosomal subunits from *Bacillus stearothermophilus* have revealed the presence of a tunnel of 100–110 Å in length and with a diameter of up to 25 Å [1]. A similar feature, although less clearly resolved, was also seen in reconstructed models of 70S and 80S ribosomes [2, 3]. This tunnel may provide the path for the nascent protein chain on the ribosome during the elongation cycle of protein biosynthesis. The existence of such a tunnel has previously been suggested by biochemical experiments [4–6].

In order to study the function of this tunnel, we attempted to develop an experimental procedure by which the nascent protein chain remains attached to the 50S subunit. This complex can

then be used for the production of two-dimensional crystalline sheets and for three-dimensional image reconstructions. A comparison of the crystalline sheets from subunits with a filled tunnel with those from subunits with an empty one (as previously used [1]) is expected to show whether the tunnel does indeed provide the path for the nascent polypeptide chain.

To this end, we first tried to fill the tunnel with a polyphenylalanine chain synthesized in the poly(U) system. We found, however, that upon dissociation of the 70S ribosome and separation by sucrose gradient centrifugation into its two subunits, the radioactively-labeled polyphenylalanine chain was not strongly retained by the large subunit. Accordingly, we replaced the poly(U) by the poly(A) system. Using this latter system, we could show that, with or without cross-linking, the newly-formed polylysine

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chains remained associated with the large subunits upon dissociation of the 70S ribosomes. These subunits were used for crystallization, thereby opening the way for diffraction studies with the aim of demonstrating that the ribosomal tunnel is used as a path for the nascent polypeptide chain. In this paper, we describe the isolation of the 50S subunits containing a nascent polypeptide chain and their crystallization as two-dimensional sheets.

## Materials and methods

### Buffers

The following buffers were used: a) TMA-I: 10 mM Tris-HCl, pH 7.8, 10 mM MgCl<sub>2</sub>, 30 mM NH<sub>4</sub>Cl, 6 mM 2-mercaptoethanol; b) TMA-II: same as TMA-I, except that it contained 0.3 mM MgCl<sub>2</sub>; c) HEPES: 10 mM HEPES-HCl, pH 7.8, 10 mM MgCl<sub>2</sub>, 60 mM NH<sub>4</sub>Cl, 6 mM 2-mercaptoethanol, filtered through Millipore filters of 0.45 µm pore size; d) potassium phosphate dissociation buffer: 10 mM potassium phosphate, pH 7.5, 1 mM MgCl<sub>2</sub>, 5 mM 2-mercaptoethanol.

### Growth of bacteria

*Bacillus stearothermophilus* (strain 799) was grown in a 50 l fermenter at 60°C under strong aeration to mid-log phase in L-medium containing 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 5 g of glucose, and 1 mg of MnCl<sub>2</sub> per liter of H<sub>2</sub>O. The pH was adjusted to 7.2 with NaOH.

*E. coli* K12 (strain D10) was grown at 37°C in the same L-medium as that used for *B. stearothermophilus* but without the addition of MnCl<sub>2</sub>.

### Ribosomes and ribosomal subunits

The bacteria were disrupted by grinding with Alcoa aluminium oxide powder in a mortar. *E. coli* ribosomes were extracted from the paste with potassium phosphate dissociation buffer, whereas ribosomes from *B. stearothermophilus* were extracted with TMA-I buffer. The ribosomal subunits were separated by sucrose gradient centrifugation in a Beckman Ti15 zonal rotor overnight at 25 000 rpm.

### Polylysine synthesis

The poly(A)-dependent *in vitro* synthesis of polylysine was performed essentially as described [7] for the *in vitro* synthesis of poly(U)-directed polyphenylalanine, with the exception that poly(U) was replaced by poly(A) and [<sup>14</sup>C]phenylalanine by [<sup>14</sup>C]lysine. To obtain a high level of polylysine formation, 30S ribosomal subunits from *E. coli* were used together with 50S subunits from *Bacillus stearothermophilus*

[8]. The *E. coli* 30S subunits were much more active in the *in vitro* system than those from *B. stearothermophilus*. The S<sub>150</sub> supernatant was also prepared from *E. coli* [9].

The composition of the incubation mixture (1.0 ml) was as follows: 19 mM Tris-HCl, pH 7.6, 16 mM MgCl<sub>2</sub>, 70 mM NH<sub>4</sub>Cl, 2.7 mM ATP, 0.09 mM GTP, 8.9 mM phosphoenolpyruvate, 53 µg of pyruvate kinase, 3.5 mg of tRNA<sup>*E. coli*</sup>, 0.6 mg of poly(A), 0.059 mM L-lysine, 0.15 µCi of [<sup>14</sup>C]lysine with a specific activity of 340 mCi/mmol, 5 mM 2-mercaptoethanol, 18 A<sub>260</sub> units of 30S from *E. coli*, 36 A<sub>260</sub> units of 50S ribosomal subunits from *Bacillus stearothermophilus* and 260 µl of the S<sub>150</sub> fraction from *E. coli*. The pH of the incubation mixture was 7.5.

The reaction was carried out in 1 ml aliquots for 60 min at 37°C and then cooled in ice. This was followed by centrifugation for 2 h at 20 000 rpm at 4°C in a Beckman 50 Ti rotor. The pellet was redissolved in TMA-I buffer and the solution dialyzed for 6 h at 4°C against a 500-fold vol of TMA-II. The subunits were separated by centrifugation in a linear sucrose gradient (10–35%) with TMA-II buffer in a Beckman SW27 rotor. Each tube contained 75 A<sub>260</sub> units of ribosomal subunits. Centrifugation was for 16 h at 18 000 rpm and 4°C. The fractions were collected and the absorption was monitored at 254 nm.

The fractions containing the 50S subunits were pooled and the Mg<sup>2+</sup> concentration raised to 10 mM. The subunits were pelleted either by precipitation with 10% polyethylene glycol 6000 or by centrifugation for 20 h at 42 000 rpm and 4°C in a 50 Ti rotor. The pellet was redissolved in HEPES buffer and dialyzed against the same buffer.

### Cross-linking

After incubation in the poly(A) system the ribosomes were pelleted by centrifugation in a 50 Ti rotor and redissolved in TEA-buffer containing 20 mM triethanolamine (adjusted to pH 8.0 with HCl), 50 mM KCl, 10 mM MgCl<sub>2</sub> and 10 mM dithiothreitol. After dialysis overnight against the TEA-buffer, the ribosomes (40 A<sub>260</sub>/ml) were treated with diepoxybutane (10–500 mM) for 2 h at 37°C. The reaction was terminated by adding a 4-fold molar excess of NH<sub>4</sub>Cl over diepoxybutane. The ribosomes were dialyzed against TMA-II buffer overnight at 4°C and loaded onto a sucrose gradient (10–35%) in TMA-II buffer. The ribosomal subunits were separated by centrifugation for 16 h at 4°C in a SW40 rotor at 19 000 rpm. Aliquots of the subunits were taken for determination of [<sup>14</sup>C]-labeling and of A<sub>260</sub>.

Alternatively, the cross-linking was performed with 2-iminothiolane. In this case, the reaction and isolation of the ribosomal subunits were the same as just described for diepoxybutane with the exception that the concentration of the ribosomes was 25 A<sub>260</sub>/ml and that of the reagent was 0.5–3.0 mM. The stepwise addition of the reagent followed the procedure previously described [10].

### Two-dimensional crystalline sheets

The procedure for the production of crystalline sheets of *B. stearothermophilus* 50S ribosomal subunits containing the polylysine chains was the same as that previously described [11, 12] for the production of crystalline sheets of 70S ribosomes and of 50S subunits from *B. stearothermophilus*.

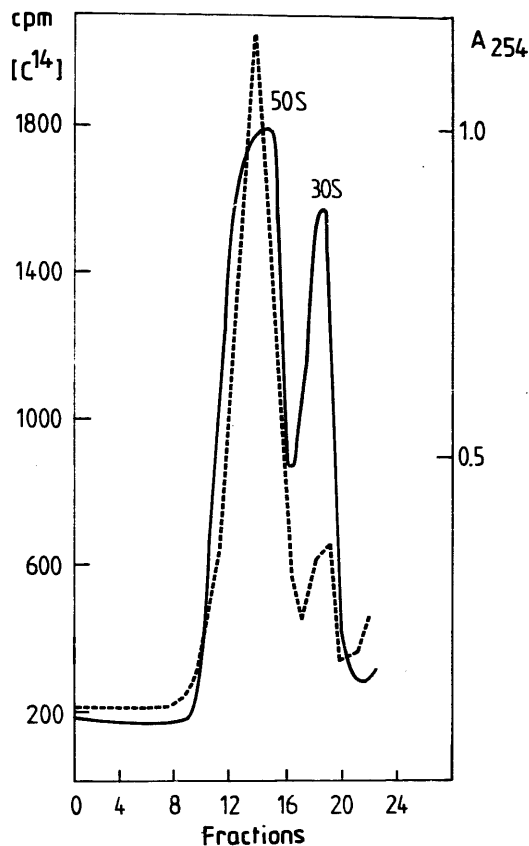
## Results and Discussion

The aim of the experiments described in this paper was to obtain 50S subunits of *B. stearothermophilus* containing a nascent polypeptide chain, and to use these subunits for crystallization, the first step in diffraction studies. As mentioned in the Introduction, we aim to compare the models obtained by image reconstruction studies of 50S subunits in which the tunnel is filled or empty. This comparison should show whether this tunnel indeed provides the path for the nascent protein chain formed during protein biosynthesis.

Our initial experiments to isolate *B. stearothermophilus* 50S ribosomal subunits to which polyphenylalanine chains synthesized in a poly(U)-programmed *in vitro* system remained attached, gave ambiguous results. We often found that the [<sup>14</sup>C]-labeled polyphenylalanine chain was released from the 50S subunit after dissociation of the 70S ribosome and isolation of its subunits by sucrose gradient centrifugation. Various modifications of the procedure for dissociation of the ribosomes and for isolation of the subunits did not improve this situation (data not shown).

Accordingly, we tried to cross-link the nascent polypeptide chain to the 50S ribosomal subunits with bifunctional reagents. Since phenylalanine is an inert amino acid for cross-linking, we replaced the poly(U) by the poly(A) system, which codes for polylysine. The ε-amino group of the lysine side chain readily reacts with bifunctional reagents, such as 2-iminothiolane. Cross-linking was employed after synthesis of the polylysine chain in the poly(A) system and the subunits were separated by sucrose gradient centrifugation. It was indeed found that the 50S ribosomal subunits contained the [<sup>14</sup>C]-labeled polylysine chains. A similar result was also obtained in control experiments without cross-linking. Therefore, we omitted the cross-linking step in the later studies.

As shown in Fig. 1, the [<sup>14</sup>C]-labeled polylysine chains migrated with the 50S ribosomal subunit. A calculation showed that, on the aver-



**Fig. 1.** Separation of ribosomal subunits by sucrose gradient centrifugation. After incubation in the poly(A) system, the ribosomes were dialyzed against TMA-II buffer and centrifuged on a sucrose gradient. Radioactivity (broken line) and  $A_{254}$  were measured.

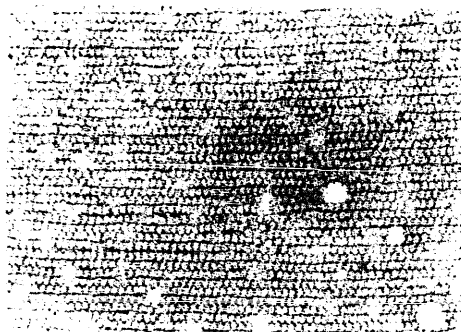
age, each subunit contained a polypeptide of 15–20 lysine residues. This value is about 30% lower than that calculated for the length of the polylysine chain per 70S ribosome before dissociation into subunits. Therefore, it is likely that about 30% of the ribosomes have lost their polylysine chains during the dissociation into the subunits and / or their separation by sucrose gradient centrifugation.

The fact that we reproducibly obtained 50S subunits to which polylysine (but not polyphenylalanine) chains were attached indicates that the subunit has a higher affinity for positively charged than for neutral amino acids. If the nascent peptide does indeed pass through the tunnel, then a possible explanation would be that a significant proportion of the walls of the tunnel

are composed of RNA. It would be interesting to confirm this hypothesis by additional experiments.

After sucrose gradient centrifugation, the fractions containing the 50S subunits (Fig. 1) were pooled, and the  $Mg^{2+}$ -concentration was raised to 10 mM. The subunits were pelleted by ultracentrifugation and used for the production of two-dimensional crystalline sheets, an example of which is shown in Fig. 2. It was found that no ordered sheets could be obtained when the 50S subunits were precipitated by polyethylene glycol after sucrose gradient separation, as opposed to pelleting by ultracentrifugation.

We are able reproducibly to obtain crystalline sheets of 50S subunits with as yet undetermined quantities of nascent polylysine chains in sufficient amounts and in reasonable order (40–



**Fig. 2.** An electron micrograph of a two-dimensional sheet of 50S subunits negatively stained with 1% gold thioglucose.

45 Å). Attempts to improve the order and to perform image reconstruction studies are now in progress.

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