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Formation and crystallization of *Thermus thermophilus* 70S ribosome/tRNA complexes

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70S ribosomes from *Thermus thermophilus* are able to form ternary complexes with *N*-AcPhe-tRNA^{Phe} from either *Thermus thermophilus* or *Escherichia coli*, in the presence of a short oligo(U) of six or nine uridines. A complex of *N*-AcPhe-tRNA^{Phe}/$(U)_9$/70S ribosome from *Th. thermophilus* was crystallized under the same conditions used for the growth of crystals from isolated ribosomes (S.D. Trakhanov, et al., (1987) FEBS Lett. 220, 319–322).

Ribosome; tRNA-binding; Crystallization

1. INTRODUCTION

Recently, large well-ordered three-dimensional crystals of 70S ribosomes from *Thermus thermophilus* were obtained [1]. Preliminary X-Ray studies of these crystals have been done [2] and X-Ray data could be collected to 18 Å resolution. Functional complexes of 70S ribosomes from *Th. thermophilus* together with an average of 1.5–1.8 equivalents of Phe-tRNA^{Phe} from *E. coli* and a RNA chain composed of 35 ± 5 uridines [3] have been obtained and X-Ray data were collected up to 15 Å resolution.

The big size of the ribosome (molecular weight 2 300 000) dictates the use of an extremely heavy and dense group for phasing. A gold cluster that contains 11 atoms of gold metal under organic surface seems to be the most suitable for derivation of ribosomal particles or non-ribosomal components capable to form complexes with ribosomes.

In this paper we present a new crystal obtained from a complex between 70S ribosomes from *Th. thermophilus*, with *N*-Ac[^{14}C]Phe-tRNA^{Phe} and $(U)_9$. This complex can be used to prepare derivatives containing either *N*-AcPhe-tRNA^{Phe} or oligo(U) modified by heavy atom clusters.

2. MATERIALS AND METHODS

2.1. *Ribosomes*

Ribosomes from *Thermus thermophilus* cells were purified according to [4]. The purity and the integrity of the final preparations were tested by the determination of their sedimentation coefficient, and of their protein content by two-dimensional SDS-PAGE [5]. Functional activity was tested in a poly (U)-directed cell-free system (see below).

2.2. *Cell-free translation system from* Th. thermophilus

The preincubation mixture (25 μl) consisted of 3.5 pmol of 70S ribosomes, 100–150 μg of total tRNA from *Th. thermophilus* containing 80–120 pmol [^{14}C]Phe-tRNA [4], 50 μg of poly(U), 20 mM $MgCl_2$, 100 mM KCl, 20 mM MOPS/KOH pH 7.5, 1 mM dithiotreitol. The mixture was incubated at 25°C for 5 min [6]. The reaction was started by adding the other components to the reaction mixture (50 μg of fraction S100 from *Th. thermophilus* [4], 0.3 μmol ATP, 40 nmol GTP, 0.5 nmol [^{14}C]phenylalanine (sp. act. 513 mCi/mmol, Amersham). The final volume was 50 μl. Incubation was at 65°C. Incorporation was estimated by measuring the radioactivity in hot trichloracetic acid- insoluble materials.

2.3. N-*acetyl*[^{14}*C*]*Phe-tRNA*^{Phe} *from* Escherichia coli *and* Thermus thermophilus

Acylation of the commercial preparation of tRNA^{Phe} from *E. coli* (Boehringer-Mannheim, 1016 pmol/*A*260 unit) by [^{14}C]phenylalanine (Amersham, 513 mCi/mmol) was done using phenylalanyl-tRNA synthetase from yeast [7]. The specific activity of the phenylalanyl-tRNA synthetase (kindly provided by Dr P. Remy, Strasbourg) was 2700 U/mg protein. The resultant [^{14}C]Phe-tRNA^{Phe} was obtained with a specific activity of 750 pmol/*A*260 unit. *N*-Ac[^{14}C]Phe-tRNA^{Phe} was obtained by treatment with acetosuccinimide [8]. In order to remove uncharged tRNA^{Phe}, the acetylation product was chromatographed on a BD-cellulose column [9]. The final product contained 1650–1700 pmol *N*-acetyl-phenylalanine/*A*260 unit.

tRNA^{Phe} from *Th. thermophilus* was aminoacylated by the enzyme from *E. coli* with [^{14}C]phenylalanine [4] within a total tRNA preparation. The [^{14}C]Phe-tRNA^{Phe} was then isolated by affinity chromatography [10]. A specific activity of [^{14}C]Phe-tRNA^{Phe} of 900–1000 pmol/*A*260 unit was obtained *Th. thermophilus N*-Ac[^{14}C]Phe-tRNA^{Phe} was prepared as described above at a final specific activity of 1550–1600 pmol *N*-Ac[^{14}C]Phe/*A*260 unit of tRNA.

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2.4. *Formation and analysis of tRNA/70S ribosome complexes*

For the binding experiments, the standard buffer was used: 20 mM MOPS/KOH pH 7.5, 100 mM KCl, 1 mM dithiothreitol and various concentrations of magnesium chloride, as indicated.

Binding of *N*-Ac[^{14}C]Phe-tRNA^{Phe} from *E. coli* or *Th. thermophilus* was performed as follows. First, 7 pmol of ribosomes were incubated with 10 μg of poly(U) or 0.2 μg of $(U)_6$ or $(U)_9$ in 20 μl of standard buffer for 5 min at 37°C. The templates of oligo(U) were a gift of Dr G.G. Karpova (Novosibirsk). Then, 14 pmol of *N*-Ac[^{14}C]Phe-tRNA^{Phe} were added to 20 μl of the same buffer. The reaction mixture was incubated at 37°C or 65°C for 20 min. The formation of tRNA/ribosome complexes was measured by applying the reaction mixtures to nitrocellulose filters (Millipore, HA-filters [11]. The standard puromycin assay was performed by the addition of 7.5 μl of 10 mM puromycin followed by incubation for 20 min at 4°C. The extraction procedure of *N*-acetyl[^{14}C]Phe-puromycin was conducted as described [12].

2.5. *Crystallization*

In order to crystallize ribosome complexes with *N*-Ac[^{14}C]Phe-tRNA^{Phe}, aliquots of the reaction mixture containing 500 pmol of ribosomes and corresponding amounts of the other components in standard buffer with 40 mM $MgCl_2$ were taken. The incubation temperature was 65°C. After incubation, the reaction mixture was concentrated by centrifugation in Centricon 30, until a concentration of 10 mg/ml of ribosome was reached. In order to perform the functional test we needed large amount of microcrystals. For this purpose crystallisation was performed by dialysis using 2-methyl 2,4-pentanediol (MPD) as a precipitant. For growing large cystals the hanging drop method was used.

2.6. *Testing the nature of the crystallized material*

The microcrystals were collected by centrifugation from the mother liquid containing standard buffer with 40 mM $MgCl_2$ and 15% MPD as a precipitant. Then, the microcrystal pellet was washed several times with the same buffer containing 10% MPD. The microcrystals were stable under these conditions. Washed crystals were dissolved in buffer A containing 10 mM $MgCl_2$ at 37°C. To estimate the ribosomal activity, the dissolved sample was tested for binding of ribosomes to nitrocellulose filters and for the puromycin reaction as described above.

Table I

Binding of *N*-Ac[^{14}C]Phe-tRNA^{Phe} to 70S ribosomes

Experiments	tRNA bound, (pmol %)	Puromycin reactivity, (pmol %)
No. I*		
$Rs_{Th.\ therm}$ + p(U)+		
N-Ac[^{14}C]Phe-tRNA$^{Phe}{}_{E.\ coli}$		
(a) at 15 mM $MgCl_2$	1.40 (20%)	
(b) at 20 mM $MgCl_2$	1.75 (25%)	
(c) at 30 mM $MgCl_2$	2.80 (40%)	ND
(d) at 40 mM $MgCl_2$	3.50 (50%)	
(e) at 50 mM $MgCl_2$	2.73 (39%)	
No. II		
$Rs_{Th.\ therm.}$+ p(U)+		
N-Ac[^{14}C]Phe-tRNA$^{Phe}{}_{Th.\ therm}$		
(a) incubation 20 min at 37°C	3.20 (45%)	3.13 (97%)
(b) incubation 20 min at 65°C	4.75 (68%)	4.65 (98%)
No. III		
1. $Rs_{Th.\ therm.}$+ p(U)+		
(a) *N*-Ac[^{14}C]Phe-tRNA$^{Phe}{}_{Th.\ therm.}$	4.50 (64%)	4.41 (91%)
(b) *N*-Ac[^{14}C]Phe-tRNA$^{Phe}{}_{E.\ coli}$	4.76 (68%)	4.76 (100%)
2. $Rs_{E.\ coli}$+ p(U)+		
(a) *N*-Ac[^{14}C]Phe-tRNA$^{Phe}{}_{Th.\ therm.}$	6.16 (88%)	6.16 (100%)
(b) *N*-Ac[^{14}C]Phe-tRNA$^{Phe}{}_{E.\ coli}$	6.65 (95%)	6.12 (92%)
No. IV		
$Rs_{Th.\ therm.}$+		
N-Ac[^{14}C]Phe-tRNA$^{Phe}{}_{E.\ coli}$		
(a) +p(U)	4.55 (65%)	4.64 (102%)
(b) +$(U)_9$	4.41 (63%)	4.28 (97%)
(c) +$(U)_6$	4.13 (59%)	3.92 (95%)

tRNA binding is expressed as the amount of tRNA bound/7.0 pmol 70S ribosomes, using a standard filter assay (see section 2). Puromycin reactivity is expressed as the number of pmol of puromycin-reactive tRNA/7.0 pmol ribosomes. Experiments II to IV were performed with 40 mM $MgCl_2$. *Incubation was 20 min at 65°C.

3. RESULTS AND DISCUSSION

It turned out that preparations of ribosomes with equal physico-chemical parameters may differ in functional activity in cell-free systems. We used the cell-free translational system from *Th. thermophilus* which was optimized by adjusting the concentration of all components [4]. The maximal efficiency of polyphenylalanine synthesis on the ribosomes from *Th. thermophilus* in the poly(U)-dependent cell-free translational system is 5–6 pmol of [^{14}C]phenylalanine per 1 μg ribosomes (Fig. 1). This value does not differ from the synthesis efficiency of the *E. coli* system. The most efficient ribosome preparation in the cell-free system was used for the formation of complexes of *Th. thermophilus* 70S ribosomes with message and tRNA (see section 2.1).

As shown in Table I (Exp. 1), the binding of *N*-Ac[^{14}C]Phe-tRNA^{Phe} to *Th. thermophilus* 70S ribosomes is dependent on the Mg^{2+} concentration. The most efficient binding was obtained with a concentration of 40 mM $MgCl_2$. All studies on the formation of the complex of 70S ribosome/mRNA/*N*-AcPhe-tRNA^{Phe} were carried out under conditions described in section 2.4 in the presence of 40 mM $MgCl_2$.

Table I also shows that the binding of *N*-Ac[^{14}C]Phe-tRNA^{Phe} increases with temperature. At 65°C in the presence of 40 mM $MgCl_2$, 60–70% ribosomes bind *N*-Ac[^{14}C]Phe-tRNA^{Phe}. Almost full reactivity towards puromycin confirmed its location in the ribosomal P-site. It is noteworthy that *N*-AcPhe-tRNA^{Phe} from both *E. coli* and *Th. thermophilus* are capable to serve as a substrate in forming the complex with ribosomes with the same efficiency (Table I, Exp. III, 1).

The data of comparative experiments with 70S ribosomes from *E. coli* are represented in Table I (Exp. III, 2). In this case, the formation of the complex between the *E. coli* 70S ribosome and tRNA was carried out under conditions described in section 2.4 at 20 mM $MgCl_2$ (with a temperature of incubation of 37°C). It was shown that *N*-AcPhe-tRNA^{Phe} from both *Th. thermophilus* and *E. coli* bind to *E. coli* ribosomes with higher efficiency than to *Th. thermophilus* ribosomes. Certainly, this difference is explained by the quality of preparation of ribosomes (80–90% of binding *N*-AcPhe-

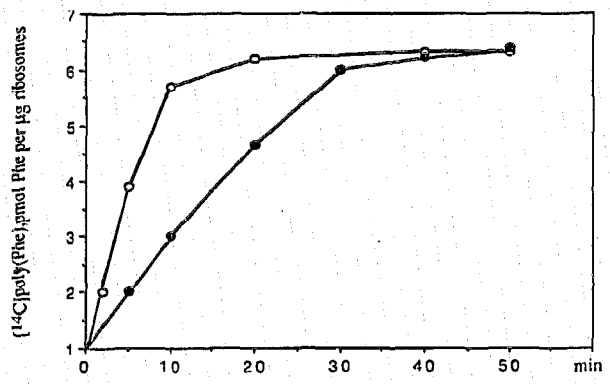

Fig. 1. Polyphenylalanine synthesis in the poly(U)-dependent cell-free translation system. (○) *Th. thermophilus* at 65°C, (●) *E. coli* at 37°C (Kolb, V.A., personal communication).

$tRNA^{Phe}$ could only be obtained with one of the preparations of *Th. thermophilus* ribosomes).

Poly(U), $(U)_9$ or $(U)_6$, were tested as templates for binding *N*-Ac-Phe-$tRNA^{Phe}$ to 70S ribosomes. As shown (Table I, Exp. IV) there is no significant difference in the efficiency of forming complexes in the presence of short oligo(U) or poly(U).

Crystallization assays of the complex 70S ribosome/*N*-Ac[^{14}C]Phe-$tRNA^{Phe}$/$(U)_9$ were conducted as described (see section 2.5). Microcrystals of the complex of *Th. thermophilus* 70S ribosome with *N*-Ac[^{14}C]Phe-$tRNA^{Phe}$ from *E. coli* and $(U)_9$ were obtained within 3–4 days under the described conditions. They were visible under a light microscope as bipyramides. Similar crystals were grown by the hanging drop method (Fig. 2).

We verified that the crystals were actually composed of the complex of ribosomes with *E. coli* *N*-Ac[^{14}C]Phe-$tRNA^{Phe}$. The dissolved crystals showed the same amount of bound molecules of *N*-AcPhe-$tRNA^{Phe}$ per ribosome before and after crystallization (55–60%). It is also important to note that *N*-AcPhe-$tRNA^{Phe}$ was still located in the ribosomal P-site (as measured by the standard puromycin reaction) in the complex from dissolved crystals.

The *Th. thermophilus* 70S ribosome/*N*-AcPhe-$tRNA^{Phe}$/$(U)_9$ complex can be prepared by the use of heavy atom cluster derivatives of tRNA or oligo(U). This approach can also be used probably in the case of other functional complexes of the 70S ribosome from *Th. thermophilus* together with an average of 1.5–1.8 equivalents of Phe-$tRNA^{Phe}$ from *E. coli* and an RNA chain composed of 35 ± 5 uridines [3]. This represents the first example of crystals obtained from a ribosome-P-site bound *N*-AcPhe-$tRNA^{Phe}$/mRNA complex with a defined stoichiometry.

In summary, these preliminary results provide evidence that microcrystals could be obtained from complexes containing the *Th. thermophilus* 70S ribosome, *N*-Ac[^{14}C]Phe-$tRNA^{Phe}$ and $(U)_9$ as a messenger. By using [^{14}C]Phe-tRNA, it was possible to verify that the same amount of complex (55–60%) persists in the microcrystal.

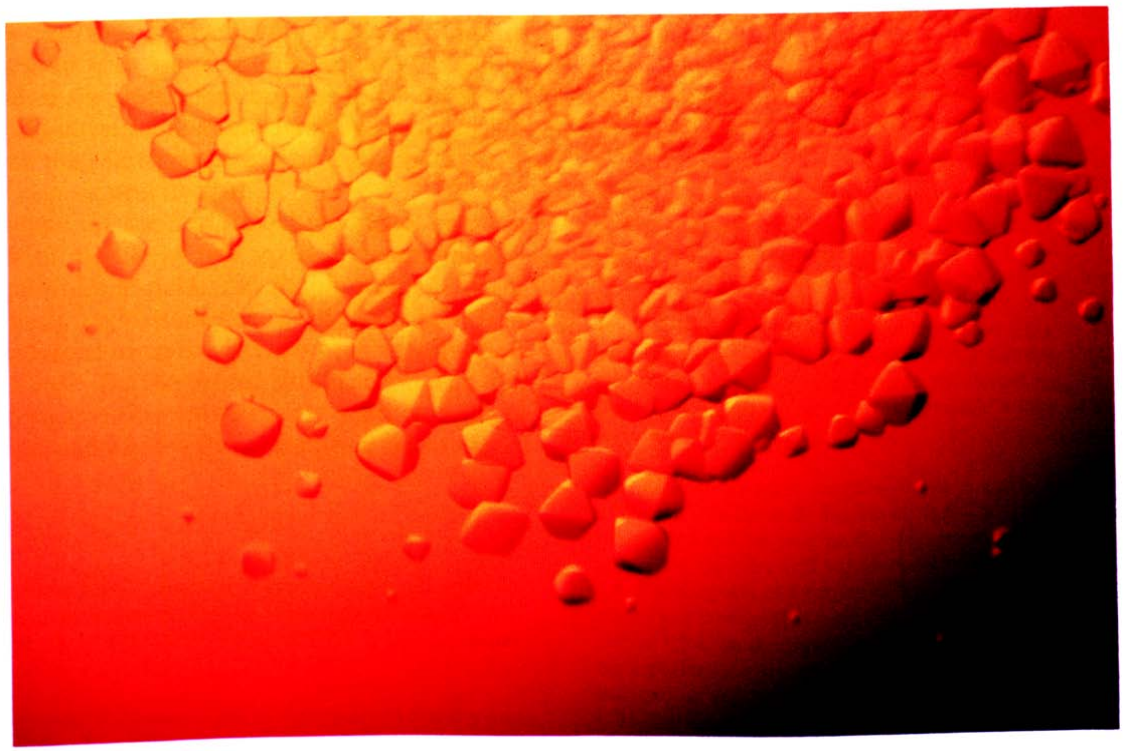

Fig. 2. Crystals of the comples 70S ribosome/*N*-Ac[^{14}C]Phe-$tRNA^{Phe}$/ $(U)_9$, obtained by the hainging drop method in standard buffer (see section 2) with 15% MPD as a precipitant.

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