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Crystals of complexes mimicking protein biosynthesis are suitable for crystallographic studies

H.A.S. Hansen [1], N. Volkmann [1], J. Piefke [2], C. Glotz [2], S. Weinstein [2,3], I. Makowski [2], S. Meyer [1], H.G. Wittmann [2†] and A. Yonath [1,3]

[1] *Max-Planck-Research-Unit for Structural Molecular Biology, Hamburg,* [2] *Max-Planck-Institute for Molecular Genetics, Berlin 33 (F.R.G.)*
and [3] *Department Structural Chemistry, Weizmann Institute of Science, Rehovot (Israel)*

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A complex of 70S ribosomes from *Thermus thermophilus* together with an average of 1.5–1.8 equivalents of PhetRNA^{Phe} and a short mRNA chain, composed of 35 ± 5 uridines, was crystallized under the conditions used for the growth of crystals of isolated ribosomes from the same source. Considering the reproducibility of their growth, their internal order and their shape, the crystals of the complex are superior to those of isolated ribosomes. In accord with previous three-dimensional reconstruction and modeling experiments, we conclude that the complex is less flexible and that an average population of complexes is more homogeneous than that of isolated 70S ribosomes. The crystals of the complex diffract to higher than 15 Å resolution and can be irradiated with synchrotron X-ray beam at cryo-temperatures for days without noticeable decay. Since the crystals of the complex are apparently isomorphous with these of the isolated 70S ribosomes ($P4_12_12$; $a = b = 526$; $c = 315$ Å), they should provide a tool for phasing as well as for locating the mRNA and tRNA binding sites.

Introduction

Many attempts have been made to grow crystals of ribosomes, the cell organelles which provide the site for biosynthesis of proteins. We first reported the growth of well ordered microcrystals of 70S ribosomes from *E. coli* [1]. Later on, larger and better crystals were obtained from 70S particles of two thermophilic eubacteria: *Thermus thermophilus* [2–5] and *Bacillus stearothermophilus* [2,5]. These crystals were of intermediate size, but consistently diffract to low resolution: 18–24 Å and 35 Å, respectively.

In parallel we were able to grow crystals of large (50S) and small (30S) ribosomal subunits which diffract to 4.5 Å and 7.3 Å, respectively [6]. Thus, we assumed that the poor internal order of the crystals of 70S particles is due to the heterogeneity of the crystallizing material, which was prepared from logarithmically growing cells. Such preparations ought to be composed of 70S ribosomes 'caught' in different conformations according to the stages of protein biosynthesis they happen to be at. A further source of disorder in the crystals of 70S ribosomes may be due to undefined amounts of various non-ribosomal components which participate in protein biosynthesis and were not removed from the ribosome throughout the preparation.

We attempted crystallization of compounds with a better defined composition using either 70S particles which were assembled in vitro from purified small and large ribosomal subunits, or complexes of ribosomes together with a few selected non-ribosomal components involved in protein biosynthesis. We first used for crystallization 70S assemblies with a high biological activity, namely a hybrid made of 30S subunits from *E. coli* and 50S subunits from *B. stearothermophilus*. These experiments led to the formation of large and well shaped crystals, which were found to contain only 50S subunits [5,7]. Thus, it was shown that even most active 70S assemblies dissociate when they are not given a chance to be involved in the biosynthetic process for relatively long periods.

An alternative strategy was to crystallize ribosomes 'frozen' at distinct functional states. These should provide homogeneous populations in which the ribosomes are annealed in relatively rigid conformations. To this end a series of complexes were designed based on biochemical and functional studies (for reviews see Ref.

Correspondence: A. Yonath, Department of Structural Chemistry, Weizmann Institute of Science, Rehovot, Israel.

8, 9) as well as on our three-dimensional image reconstructions. The latter provided us with models of 50S subunits and 70S ribosomes which were determined from tilt series of two-dimensional sheets of *B. stearothermophilus* at 28 and 47 Å respectively [6,7,10,11]. These models revealed several gross features which had not been detected previously in prokaryotic ribosomes. One of these features is an empty space, comprising of 15–20% of the volume of the 70S ribosome and located at the interface between the parts of the ribosome which were assigned by us as the two ribosomal subunits and is presumably the site of the actual process of protein biosynthesis. Another prominent feature is a tunnel of about 100 Å in length and up to 25 Å in diameter which spans the large ribosomal subunit [10]. This tunnel leads from the intersubunit free space to a location compatible with the exit site of the nascent protein chain as identified by immuno electron microscopy [12]. In accord with experiments which show that the ribosome masks the growing polypeptide [13–18] and with the existence of a similar tunnel in eukaryotic ribosomes [19], we proposed that this tunnel provides the path taken by the nascent protein chain. In addition, a groove was detected in an RNA-rich region within the 30S subunit [6,11], in a position suitable to be the mRNA binding site [20]. Model-building experiments showed that the groove and the tunnel can be bridged by a tRNA molecule, so that its anticodon end is located close to the mRNA binding site and its CCA-terminus is positioned such that the peptidyl group may extend into the tunnel (Fig. 1). In fact, the intersubunit space is large enough to accommodate three molecules of tRNA (Fig. 1) with room to spare for other factors involved in protein biosynthesis [6,21].

Despite the low resolution at which these models were reconstructed and the uncertainties concerning the spatial arrangements of the various components, we believed that there was enough justification to use our speculative conclusions for constructing sound crystallographic experiments. We assumed that the intersubunit free space contributes to the internal motion of the ribosomes. This flexibility manifests itself not only by allowing for the dynamics involved in the process of biosynthesis of proteins, but also in the poor internal order of the crystals of the 70S particles. Thus, we have designed a series of complexes, each of which mimics a stage in protein biosynthesis and contains ribosomes in which the motional freedom is limited. The key feature in this series is the mRNA chain. For the production of suitable complexes, this chain has to be of a high affinity to the ribosome and of a sequence suitable for productive initiation of biosynthesis. At the same time, it should be of a length which allows its full accommodation within the intersubunit free space. It is clear that the production of such complexes in quantities suitable for crystallographic studies, is time consuming and requires significant sophistication, the level of which is

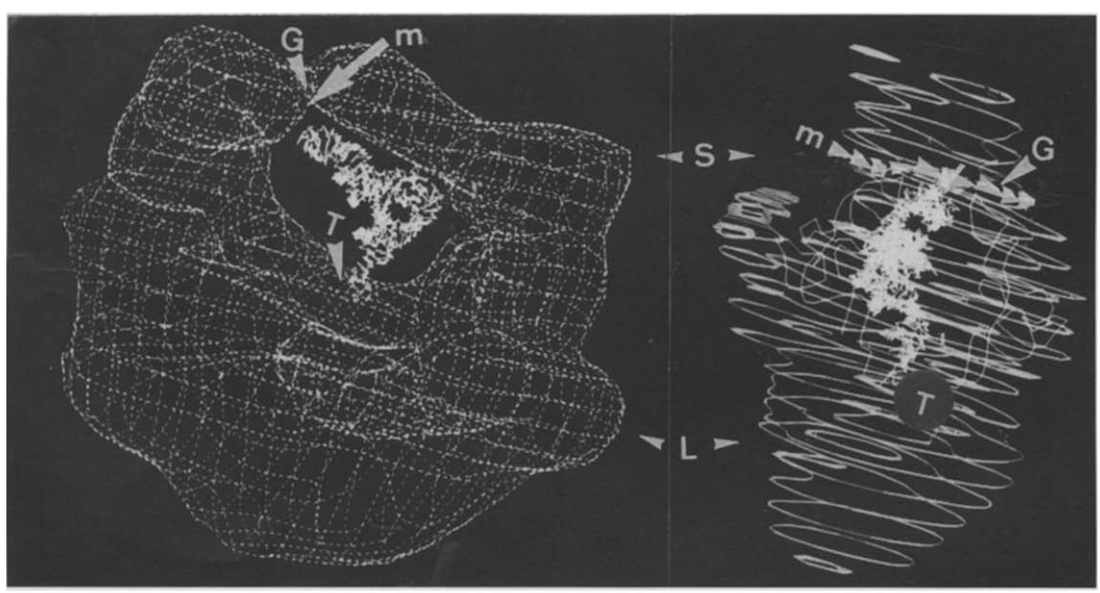

Fig. 1. Two perpendicular views, created by computer-graphics, of the outline of the reconstructed model of the 70S ribosome [6,11]. The envelope of the 70 S particle is shown as a dotted net on the left side, and as parallel lines (on the right). (S) and (L) indicate the small (30S) and the large (50S) subunits, respectively. The arrows point to the interface between the two subunits. (G) marks the groove rich in RNA in the small subunit, and (T) shows the entrance to the tunnel. 'Model built' into the intersubunit free space are: (m) a piece of 28 ribonucleotides, in an arbitrary conformation, which may simulate the mRNA chain (highlighted on the right-side figure, with arrow heads), together with three molecules of tRNA. Two of these tRNA molecules are shown as traces of their backbone. The tRNA molecule which points directly into the tunnel is highlighted by including all its atoms. For clarity we show on the left hand only the highlighted tRNA molecule.

determined by the nature of the mRNA. Thus, to assess to viability of this approach, we have designed a family of mRNA chains with increasing complexity. The simplest members in this family are homopolynucleotides of random lengths. Short homopolynucleotides with a defined length are somewhat more suitable. Thus, we prepared oligomers with pre-determined lengths by basic hydrolysis of the commercially available long poly(U) chains (containing more than 3000 bases).

Here, we describe the crystallization and the characterization of a complex, composed of 70S ribosomes from *T. thermophilus*, an oligomer of mRNA, containing 35 ± 5 uridines and an average of 1.5–1.8 equivalents of PhetRNA^{Phe}. Although this complex is of a preliminary nature, its crystals are more ordered that those of the isolated ribosomes, and we expect that the analysis of its structure will provide us with useful information concerning the mode of action of the ribosomes, the location of the mRNA chain and the tRNA molecules, as well as assist us in planning future experiments.

Experimental procedures

Intact 70S ribosomes were prepared and their homogeneity and activity were tested as described in [5].

Short segments of defined length of poly(U) were prepared by basic hydrolysis, followed by Sephacryl S100 column, according to (Grinke, A., unpublished data).

The extent of binding of PhetRNA^{Phe} (from *E. coli*) and poly(U) segments was determined by using radioactive PhetRNA^{Phe}, according to [22].

Crystals were grown within 6–10 d by vapor diffusion in hanging drops in Linbro dishes at 19°C from solutions containing 5–8 mg/ml of the complex in (H-I) buffer [23], and 2–10 mM spermidine. These were equilibrated with a reservoir of 15–18% 2-methyl 2,4-pentanediol (MPD) at the same pH. To obtain large crystals we used single seeds of micro crystals which appear spontaneously under the same conditions.

To establish that the crystals contained ribosomes, we washed them thoroughly in their stabilization solution, which contained 20% MPD and all the other ingredients of the crystallization drop and then dissolved them in the regular storage buffer, H-I [23]. The dissolved crystals were characterized by sucrose gradients and two-dimensional gels [2].

To establish that the crystals were actually composed of the complex with PhetRNA^{Phe} and poly (U), we have also grown crystals with radioactive [^{14}C] PhetRNA^{Phe}, washed them thoroughly (as above), and dissolved them and determined the relative amount of the crystallized ribosome (by absorbance at 260 nm) and of the bound PhetRNA^{Phe} (by measuring the radioactivity).

The procedures for the determination of exposed sulfhydryl groups on the surface of the ribosome, for the preparation of activated gold and iridium clusters, for binding these clusters or a model reagent, *N*-ethylmaleimide (NEM) to specific -SH groups on the surface of the ribosome and for the quantitative analysis of the modified particles are described in detail in [24]. In the particular case of 70S ribosomes from *T. thermophilus*, the binding was carried out according to that described in [24], but at 37°C, for 20–24 h.

Crystallographic studies were performed with synchrotron radiation using crystals mounted on monolayered glass spatulas [25] and maintained at a temperature of 90–110 K during the entire period of data collection. The data were collected on film using the rotation method (2–3° oscillation) with crystal-to-film distances of 300–500 mm (equipped with a helium path) and X-ray wave length of 1.57 Å. Crystallographic studies were carried out at station A1 at CHESS/Cornell Univ., U.S.A.

For the investigation by electron microscopy we used positively stained (with 2% uranyl acetate) thin sections of crystals that have been fixed in 0.2% glutaraldehyde and embedded in epon resin ERL 4206 [23].

Results and Discussion

Fig. 2 shows crystals of 70S ribosomes from *T. thermophilus* and of their complex with an average of 1.5–1.8 equivalents of PhetRNA^{Phe} and a short mRNA chain, composed of 35 ± 5 uridines. It is noteworthy that the conditions of the growth of crystals of pure 70S ribosomes were found to be suitable also for growing crystals of this complex. However, the reproducibility in obtaining crystals from the complex is 1–2 orders of magnitude higher than that from isolated 70S ribosomes. In addition, the morphology of the crystals of the complex seems to be of better quality than that of the crystals of isolated 70S ribosomes. The latter appear as bipyramids with smooth edges, whereas those of the complex are somewhat larger (typically 0.4–0.5 mm across) and have sharper edges. Preliminary crystallographic characterization shows that the crystals of the complex are apparently isomorphous with those of the isolated ribosomes. Both pack with $P4_22_12$ symmetry and unit-cell dimensions of: $a = b = 524$, $c = 315$ Å. Having the same density (1.063 g/cm^3) and assuming that the crystallized complex is similar in size to isolated 70S particles, we conclude that each asymmetric-unit contains one complex-unit.

As expected, the significant improvement in the reproducibility in crystal growth and in the shape of the crystals of the complex over those of the isolated ribosomes, was accompanied by a marked improvement in their internal order, as seen in electron micrographs of their positively stained thin sections (Fig. 3) and as

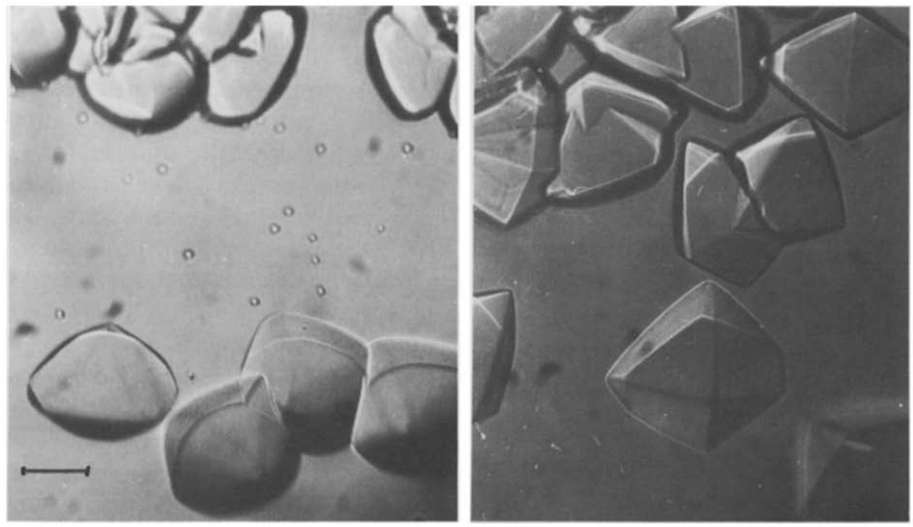

Fig. 2. Left: Crystals of 70S ribosomes from *T. thermophilus*. Right: Crystals of the complex composed of a 70S ribosome with a short chain (35 ± 5 mers) of poly(U) and an average of 1.5–1.8 equivalents of PhetRNA^{Phe}. (Bar: 0.1 mm).

reflected by the resolution of their X-ray diffraction patterns. Whereas crystals of 70S ribosomes diffract to 18–24 Å, the crystals of the complex diffract beyond 15 Å resolution (Fig. 4).

X-ray crystallographic data have been collected photographically using synchrotron beam. A common feature in the diffraction patterns of all crystals of ribosomal subunits is that the reflections of Bragg resolution higher than 18–20 Å decay within a few minutes of irradiation at 0–19 °C [25,26]. The crystals of the complex showed no exception, thus, crystallographic data to 15 Å have been collected at cryogenic temperatures and a complete diffraction data set was obtained from a single crystal. For the cryogenic pre-cooling treatment of the crystals of the complex we have employed the same procedure that we used for those of the 70S ribosomes [5]. Before cooling, these crystals were transferred to a solution which contained in addition to the crystallization buffer, 30% MPD which served as an 'anti-freeze' and at the same time increased slightly the viscosity of the solution. The crystals of this complex, similar to those of the 70S ribosomes from *T. thermophilus*, are relatively thick. Therefore, they could be mounted on single-layer spatulas [5,25]. The mounted crystals were instantaneously cooled in liquid propane, and transferred in the cooled propane to the X-ray

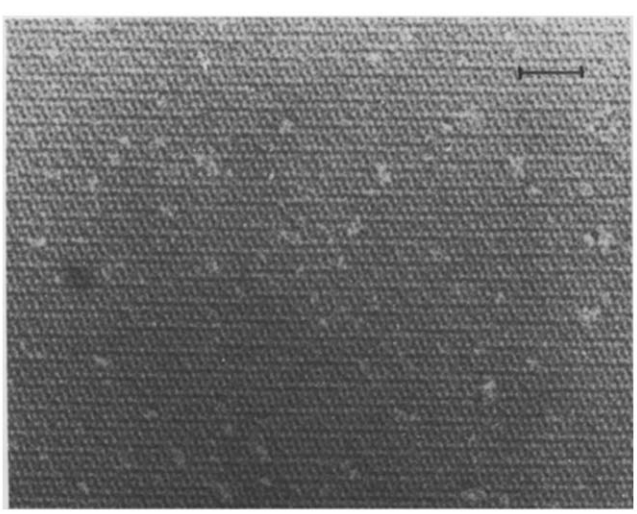

Fig. 3. An electron micrograph of a positively stained (with 2% uranyl acetate) thin section of crystals of the complex, shown in Fig. 2 (right). Bar: 2000 Å.

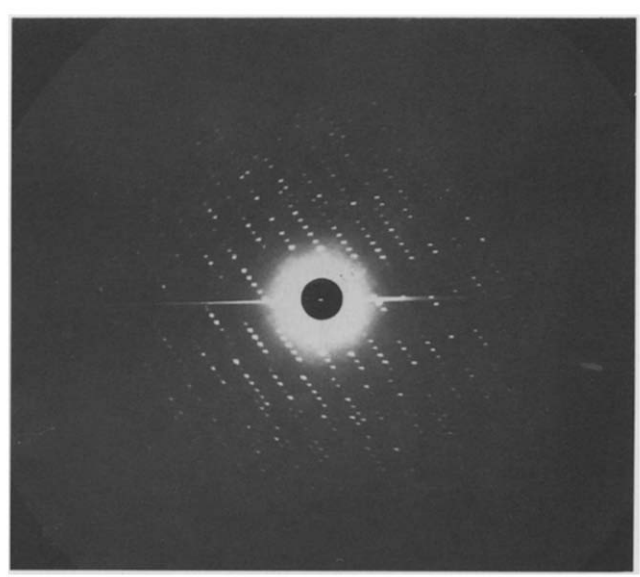

Fig. 4. 3° rotation X-ray diffraction pattern of a crystal of the complex shown in Fig. 2 (right). The pattern was obtained at 90 K at Station A1/CHESS, operating at about 5.3 Gev and 50–80 mA. The crystal-to-film distance of 500 mm was partially filled by helium. Diameter of the collimator = 0.2 mm; wave length = 1.57 Å.

camera, where they were kept in a stream of nitrogen gas (at 90–110 K) throughout the entire period of data collection.

Despite the improvement, it is clear that resolution limit of 15 Å is still far from the desired one and reflects the imperfections of the current complex. Little is known about the conditions at which the ribosomes from *T. thermophilus* bind efficiently mRNA and tRNA. Based on our preliminary experiments and extrapolating from the properties of the ribosomes from *E. coli*, we found that in solutions containing 10–15 mM Mg^{2+}, carefully prepared ribosomes, programmed with either a long or a short poly(U) chain, bind an average of 1.5–1.8 molecules of PhetRNA^{Phe}. We have produced our complex under these conditions and found that indeed, the same ratio between the tRNA and the ribosomes (i.e., 1.5–1.8 : 1) found in solution, was also kept within the crystals (see Experimental procedures).

Because in every preparation of ribosomes there is a fraction which is inactive, it is difficult to estimate the accurate stoichiometry of bound tRNA. Although the fraction of the non-bound ribosomes can be minimized, but even when it approximates zero, the quantitization of bound tRNA molecules bares some uncertainties. Thus, it is still to be determined whether an average value of 1.5–1.8 tRNA equivalents reflects our disability to measure accurately, or it indicates that even in the crystals there is some variability. This variability may contribute to the internal disorder of the crystals. Another source for the disorder of the crystals is the Poly(U) chain, which was used by us as mRNA for reasons of simplicity. Clearly, it is not the ideal messenger since it does not contain a powerful signal for attachment to the ribosome and for initiation of protein biosynthesis. Furthermore, being a homopolynucleotide, it can bind to the ribosomes at random locations along the chain, so that there may be cases at which its ends extend out of the envelop of the ribosomes and protrude into the solution in an uncontrolled fashion.

Fine tuning of ribosomes should be achieved when more sophisticated messages are introduced. Complexes with heteropolynucleotides of defined sequences which code for the specific features which are required for a tight binding to the ribosomes, are the next members in the series. The ultimate choice are complexes which contain not only mRNA and tRNA but also the nascent polypeptide chain. We have previously crystallized complexes of 50S subunits (from *B. stearothermophilus* as well as from *H. marismortui*) which contain short chains of poly(Phe) and poly(Lys) [6,27,28]. On the basis of these results we hope to grow crystals of complexes of 70S ribosomes together with a heteronucleotide which code for desirable amino-acid sequences, the corresponding loaded tRNA molecules, and a very short segment of the nascent protein chain, with a specific sequence (e.g., containing a cysteine, to which heavy atoms can be covalently bound). Since these complexes should be highly homogeneous and since they contain many elements needed to induce stability and rigidity, they should form highly ordered crystals. Therefore, we believe that the current resolution is only a temporary upper limit and it is likely to improve as we continue to identify and control the sources of the variability and the flexibility of the crystals (e.g., use more appropriate mRNA chains).

In order to estimate the individual contributions of the mRNA chain and the tRNA molecules to the stability and the rigidity of the complex, we have also attempted to co-crystallize 70S ribosomes together with the same poly(U) oligomer, but omitting the PhetRNA^{Phe}. This led to occasional growth of not very well shaped crystals. Because of the small number of available crystals from this primitive complex, we still have no idea about their internal order. However, even this semi-negative result is illuminating for the assessment of the individual contributions of the tRNA and the mRNA molecules for the construction of a stable crystallizable complex. Thus, it is clear that the presence of tRNA molecules increases the rigidity of the system, in agreement with our speculative model in which the tRNA 'locks' the two subunits together with filling up a large part of the intersubunit free space. (Fig. 1 and in Refs. 6, 21).

For the completion of the crystallographic analysis, phase information is essential. Within our efforts to phase the diffraction pattern we have extended the procedures developed by us for the derivatization of 30S ribosomal subunits from *T. thermophilus* and 50S ribosomal subunits from *B. stearothermophilus* [24]. By using a radioactive model compound, *N*-ethylmaleimide (NEM), we found conditions which enabled the covalent binding of 0.8–1 equivalents of this compound to each 70S ribosome from *T. thermophilus*, and that the radioactivity was mainly associated with one protein, temporarily named TS6.

Using the same procedure, a monofunctional undecagold cluster was attached to 70S ribosomes prior to their complexation. It was found that only half of the amount of PhetRNA^{Phe} which binds to unmodified ribosomes, could be bound to the modified ones, indicating a possible interference between the gold cluster and one of the tRNA binding sites. Unfortunately, so far, neither the modified ribosomes nor the modified complex could be crystallized.

Concluding remarks

We are aware of the speculative nature and the low resolution of the models used for the design of this complex. It also should not be forgotten that most reconstructed models from electron micrographs of negatively stained sheets, suffer from technical short-

TABLE I

Classification of the different crystals of ribosomal particles

	30S	50S	70S	Complexes of:			mutant (−L11)	
				70S + poly(U) [a]	70S + poly(U) + tRNA [b]	50S + tRNA + nascent chain [c]	50S	70S
E.c.			+					
B.st.	+	+ +	+			+	+	+
T.t.	+	+	+	+	+			
H.m.	+	+		+		+		

[a] Complex of 70S ribosomes together with an oligomer of 35 ± 5 uridines, serving as mRNA.
[b] Complex of 70S ribosomes together with an average of 1.5–1.8 equivalents of PhetRNA^{Phe} and an oligomer of 35 ± 5 uridines, serving as mRNA.
[c] Complex of 50S subunits from *H. marismortui* or *B. stearothermophilus* together with a segment of 18–20 mers of poly(Phe) or poly(Lys), respectively, and the corresponding tRNA.
B.st, Bacillus stearothermophilus; T.t, Thermus thermophilus; H.m., Halobacterium marismortui.

comings. Furthermore, the proposed fashion of binding of the m- and tRNA is based only on modeling experiments. We also realize that an improvement in the resolution of the crystals of the complex may result from other spatial arrangements. However, the mere fact that crystals which mimic a defined stage within the process of protein biosynthesis are superior to those of isolated 70S ribosomes, encourages us to proceed with our studies.

As seen in Table I, we were able to crystallize a variety of ribosomal particles. Thus, we have in hand a tool which enables investigations of the same particles from different sources, and in parallel to follow the conformational differences which take place upon the association of ribosomes from their subunits [6] as well as upon binding of components which participate in protein biosynthesis by focusing on ribosomal particles and complexes from one source. The current complexes are most suitable for this aim, since from the same organism, *T.* thermophilus, we could crystallize the 70S ribosomes [5] and the isolated small [26] and large [28] subunits. Furthermore, this complex was created in an environment containing the initiation factors as well as other compounds needed for binding of mRNA and tRNA to ribosomes [22]. Thus, although it was not explicitly shown, it is conceivable that the complex contains some of these compounds, the locations of which should be revealed once the structure of the complex is determined.

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