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Genetic and Biochemical Manipulations of the Small Ribosomal Subunit from *Thermus thermophilus* HB8

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Abstract

Crystals of the small ribosomal subunit from *Thermus thermophilus* diffract to 3Å and exhibit reasonable isomorphism and moderate resistance to irradiation. A 5Å MIR map of this particle shows a similar shape to the part assigned to this particle within the cryo-EM reconstructions of the whole ribosome and contains regions interpretable either as RNA chains or as protein motifs. To assist phasing at higher resolution we introduced recombinant methods aimed at extensive selenation for MAD phasing. We are focusing on several ribosomal proteins that can be quantitatively detached by chemical means. These proteins can be modified and subsequently reconstituted into depleted ribosomal cores. They also can be used for binding heavy atoms, by incorporating chemically reactive binding sites, such as –SH groups, into them. In parallel we are co-crystallizing the ribosomal particles with tailor made ligands, such as antibiotics or cDNA to which heavy-atoms have been attached or diffuse the latter compounds into already formed crystals.

Introduction

One of the most important and central processes in the living cell is the production of proteins according to the information encoded in the genes. The ribosome, a defined assembly of proteins and RNA translates the mRNA code to the amino acid sequence of the proteins. Active ribosomes are built of two subunits, which associate once the protein biosynthesis process is initiated. In prokaryotes the two subunits are termed 50S (large) and 30S (small). The sedimentation coefficient of the associated subunits is 70S. Two-thirds of the mass of the prokaryotic ribosomes consist of three different rRNA chains, 5S and 23S in the large subunit and 16S rRNA in the small subunit. In addition up to 60 proteins build the 70S ribosome, of which 21 belong to the small subunit.

Currently the fundamental principles of the ribosome function can be described in biochemical terms, but the detailed mechanism of the reactions it catalyzes should be understood better once its molecular structure is determined. With the knowledge of the exact three-dimensional structure of the ribosome, questions concerning the efficiency, the fidelity and the control of biosynthesis may be answered. Towards this goal crystallographic studies using synchrotron radiation are being performed on ribosomes from thermophilic and halophilic sources. The whole ribosomes of *Thermus thermophilus* (T70S) as well as their complex with mRNA and aminoacyl-tRNA crystallize well, but diffract to low resolution of 8-12Å. The subunits, namely T30S and T50S, reach 3.0Å resolution in their diffraction patterns (1-5).

Multiple isomorphous replacement (MIR), one of the methods for solving the phase problem in crystallography, requires the introduction of heavy atoms into the

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crystal and data collection from several crystals. The alternative approach, multi wavelength anomalous dispersion (MAD) is preferred in ribosomal crystallography as it frees the measurements from comparisons between diffraction patterns originating from different crystals, thus overcoming the low isomorphism of the ribosomal crystals. This method exploits the anomalous signals and their wavelength dependence originating from scatterers (e.g. selenium) introduced into the molecule. These scatterers may also be used as reliable markers after phasing. However, since the anomalous signals are significantly lower than the differences in structure factors used by the MIR method, maximum binding is desired. We therefore introduced recombinant methods for extensive selenation as well as for the insertion of potential heavy atom binding sites, such as sulfhydryl groups.

So far the derivatization of T30S crystals was performed by soaking, by covalent binding prior to the crystallization and by post-crystallization cDNA hybridization. These led to a 7.2Å map that was subsequently extended to 5Å resolution (6), showing the overall structure of the T30S particle with a morphology similar to that obtained by cryo-EM reconstructions of this subunit at its functionally active conformation. Details of these maps include elongated dense regions traceable as RNA chains and globular patches into which several known structures of ribosomal proteins could be fitted. In this manuscript we describe our approach aimed at phasing at higher resolution.

Materials and Methods

Crystallization was performed as described earlier (7). Specific mercuration of cDNA oligomers is described in (8).

All the buffers that were used are described in the Appendix.

Splitting Ribosomal Proteins from the Small Subunit:
Successful detachment of proteins from the T30S was performed in a solution of $H_{10}M_{10}N_{60}$ buffer, under two different conditions: a) 4 M LiCl and 100 mM $MgCl_2$; b) 2 M $MgCl_2$. The samples were left for 12 hours at 4°C. Separation of the split proteins from the ribosomal cores was achieved by centrifugation (Beckmann, TL – 100, 4°C at 75000 rpm for 3 hours). The supernatant, containing the detached proteins was dialyzed against $H_{10}M_{10}N_{60}$. For reconstitution, the protein depleted ribosomal cores and the split proteins were mixed in a ratio of 1:1.5 equivalents (core: split-proteins) in the reconstitution buffer (60°C for 60′). The reconstituted particles were again collected by centrifugation on TL-100 as, described above. To analyze whether the detached proteins reconstitute into their core after FPLC treatment, the split protein solution was loaded into a resourceS FPLC column and eluted with a steep gradient from 50 mM to 1 M NaCl both in 20 mM Tris-HCl pH 7.5 in 20 minutes. The eluent was collected, concentrated and reconstitution was attempted as described above.

To identify and separate the protein TS5 from the other split proteins, a mixture of split proteins was loaded into a resourceS FPLC column. Each peak was collected and assigned by its location on a 2-dimensional PAGE (9). We could identify various split proteins (i.e.TS5).

Cloning of TS5:
For the preparation of the genomic DNA *Th. thermophilus* cells were grown in "Thermus thermophilus HB8" medium (10,11), at 75°C O/N. The cells were suspended in Lysis Buffer and were incubated O/N at 37°C. The DNA was recovered by phenol-chloroform-extraction and ethanol precipitation. PCR was used for amplification of the DNA coding for the TS5-protein. Two oligonucleotides were designed, according to the known sequence of the gene (12). The upper (I) and lower (II) primers used for amplification of the TS5 gene are shown. The *Nde* I and

the *BamH* I from primers I and II, are respectively underlined.
• I: 5′-GGGAATTCCATATGCCGGAGACCGACTTTGAAG-3′ (33 nucleotides)
• II: 5′-CGGGATCCGCTCTTCACCAGCTTAACC-3′ (28 nucleotides).

For the overexpression the TS5 DNA gene was cloned into the pET-12a vector via the *Nde* I and *BamH* I cloning sites. The resulting plasmid was transformed into *E. coli* BL21 F-, ompT, rB-, mB-, (DE3) pLysS, Cmr and *E. coli* B834 F-, omp T, hsdS B, (rB- mB-), gal dcm met, (DE3) pLysS, (camR) (Novagen.). The latter strain is a methionine auxotrophe.

Overexpression of TS5 in E. coli BL21 (DE3) pLysS:
Overexpression was performed in LB-medium containing 34 μg/ml of chloramphenicol and 50 μg/ml of carbenicillin. The cells were grown at 37°C at 170 rpm. When the optical density reached A_{560} = 0.6 the culture was induced with 0.5 mM IPTG. After induction the culture was grown for three more hours. The bacteria were isolated from the medium by centrifugation at 5000 rpm for 10 minutes at 4°C.

Overexpression of TS5 in B834(DE3)pLysS:
Starter culture was grown in LB under selective pressure of chloramphenicol and carbenicillin until the optical density reached A_{560} = 0.6. The cells were collected by centrifugation at 5000 rpm, 4°C for 5 minutes and were washed twice with M9 medium. The bacteria were resuspended in M9 medium, which was supplied with 160 μg/ml of nineteen amino acids of the basic set and seleno-methionine instead of methionine. Chloramphenicol, carbenicillin and 1.6% sterile glucose were added. This culture was grown for 15 hours and then used for inoculation of the overexpressing culture in M9 with the same additives as described above. After nine hours of growth the optical density reached A_{560} = 0.63. The culture was induced with 0.5 mM IPTG. The cells were grown until the culture reached the stationary phase four hours after induction. Overexpression of proteins was analyzed by 15% SDS-PAGE.

Purification of TS5:
After overexpression the cells were collected by centrifugation (5000 rpm, 10′, 4°C) and resuspended in a resuspension buffer. Throughout the whole procedure protease inhibitors (PMSF, benzamidine) were added. The lysed cells were sonicated and centrifuged for 30 minutes at 15000 rpm. The supernatant containing the TS5 was centrifuged for 2.5 hours at 35000 rpm. After centrifugation the salt concentration in the protein solution was decreased by dialysis against Dialysis buffer F (cut-off 3500 Da). Ion exchange chromatography was used to separate TS5 from the protein mixture. First anion exchange at pH 7.5 (DEAE Sepharose) was used. TS5 does not bind to the column as its pI is above 7.5. The second step was a cation exchange with FPLC using a 1 ml resourceS column (Pharmacia). A linear salt gradient was used: from 20 mM Tris-HCl pH 7.5, 7 mM mercaptoethanol 0.1 mM benzamidine, 0.1 mM PMSF and 50 mM NaCl to the same buffer with 1 M NaCl in 30 minutes with a flow rate of 1 ml/min. The protein was eluted at about 0.45 to 0.48 M NaCl and was found to be at least 95% pure according to SDS-PAGE analysis. Additional purification with HPLC was performed for analytical purposes only, using an AQUAPORE RP-300, 7 μm, 0.4X10 cm Brownlee column. The gradient was as follows: starting with 20% ispropanol in water to 80% isopropanol with 0.1% TFA throughout, in 30 minutes. Flow rate was 1 ml/min.

Amino-acid-analysis indicated that about 7 mg selenated TS5 were purified per liter medium. This is a lower quantity than that obtained in an average, normal overexpression, probably because seleno-methionine is a poisonous substance.

Mutating TS5:
The methionine coding tails were introduced in the gene via PCR. Three additional oligos were synthesized, with the added methionine codons either at the N-ter-

minus next to the initial AUG of the TS5 gene (oligos I and II) or at the C-terminus of the protein (oligo III).
I: 5′-GGAATTCCATATGAAAATGATGATGATGATGCCGGAGACCGACTTTGAAG-3′ (N-terminus, five methionine)
II: 5′-GGAATTCCATATGAAAATGATGATGCCGGAGACCGACTTTGAAG-3′ (N-terminus, three methionine)
III: 5′-CGCGGATCCGCGTTACATCATCATACCTTGAGCCTGGGCATGGGC-3′ (C-terminus, three methionine)

The same PCR conditions as for the native TS5 were used. The reaction was successful for the pair oligo I and the native TS5 lower primer, for oligoII with oligoIII and for oligo III with the native TS5 upper primer. PCR with the first and the third oligo did not produce a correct sequence. The obtained DNA fragments were cloned into the pET12a plasmid and transformed into *E. coli* BL 21 (DE3) pLysS and *E. coli* B834 (DE3) pLysS as described above for the native protein. The overexpression was successful, although just very small amounts of overexpressed protein were obtained.

Labeling of T30S with cDNA:

Labeling by Non-Modified Oligonucleotides:
In order to analyze the hybridization of the synthesized oligonucleotides to the complementary region of the rRNA, we first used non-modified oligonucleotides. These were phosphorylated at the 5′ end using (^{32}P)-γ-ATP and the binding was checked by sucrose gradient centrifugation as follows: a ten molar excess of oligonucleotides was incubated with the T30S subunits at 40°C for 30 minutes and then the solution was left to reach room temperature slowly. The reaction mixture was loaded onto a sucrose gradient 5-20% in $H_{10}M_{10}N_{60}$ (pH 7.8) and centrifuged at 22000rpm for 10hrs in a Beckman SW40 rotor. The absorbence at 260nm and the radioactivity (^{32}P) of the collected fractions were monitored. The amount of bound oligomers was calculated from the amount of radioactivity that co-migrated with T30S on the gradient. The unbound oligomers were found at the top of the gradient.

Labeling of the Oligomers with 3Cys-TAMM:
TAMM was used as a heavy atom derivative for the cDNA oligomers. In order to avoid crosslinking, three of the four mercury atoms in the TAMM molecule were blocked before labeling the oligomers. For this aim TAMM (1mM) was incubated with a three molar excess of (^{35}S)-cysteine in 1% acetic acid. The oligonucleotides were phosphorylated using T4 polynucleotide kinase followed by the labeling with TAMM. The radioactive label is also useful for monitoring the binding of the TAMM modified oligomers to the T30S subunits. A 1.3 molar excess of the 3Cys-TAMM complex was used for each 6-Thio-dG in the oligonucleotide sequence. The reaction was performed at room temperature and the unbound 3Cys-TAMM complex was separated from the oligomers through NAP-column filtration.

The labeling of the oligonucleotides was monitored by the disappearance of the peak characteristic to the SH of the 6-Thio-dG at 340nm as well as by the co-migration of (^{35}S)-cysteine with the oligonucleotide peak in an urea-acrylamide-gel 20% in TB buffer. The TAMM-oligonucleotide migrates differently than the non-modified one (Figure 1)

Binding the Labeled Oligo to the T30S:
The binding of the 3Cys-TAMM labeled oligonucleotides to T30S was checked in the same way as the non-modified oligonucleotide (Figure 1).

Modification of detachable ribosomal proteins:

Selenium was found suitable for MAD phasing of many biological macromole-

cules. It is introduced into the ribosome by substitution of methionine by seleno-methionine. Since *Thermus thermophilus* does not grow on seleno methionine and totally reconstituted ribosomal particles did not yield highly diffracting crystals, we focused on proteins that can be quantitatively and reproducibly detached from native T30S by chemical means. The methionine auxotrophe of *E. coli* that grows on seleno-methionine despite its toxicity (13), allowed us to overexpress T30S pro-

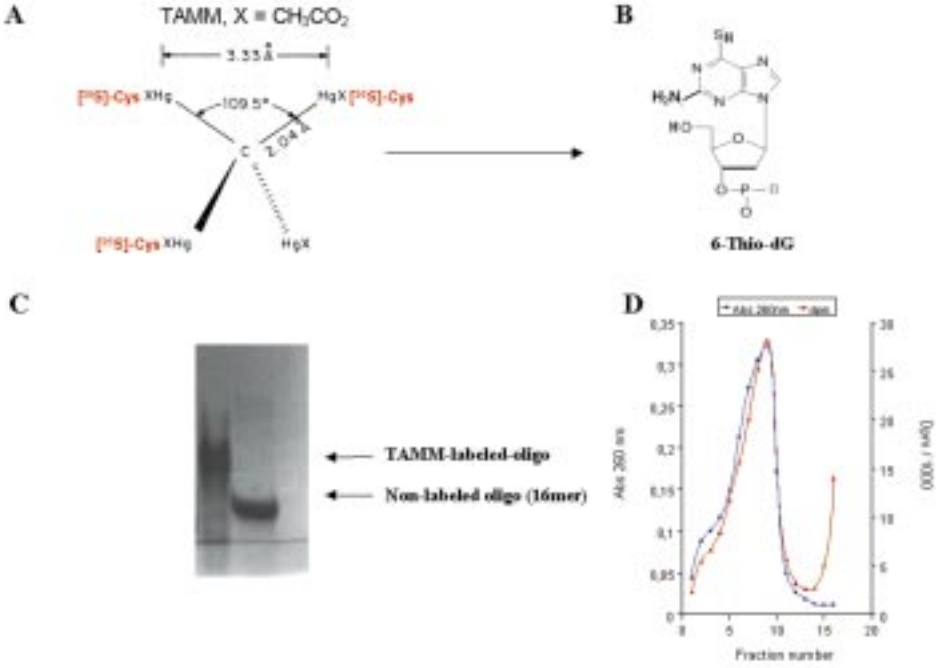

Figure 1: TAMM labeling of DNA-oligonucleotides. A. TAMM was incubated with a three molar excess of ^{35}S-Cys in order to prevent crosslinking and to be able to follow the labeling reaction. B. The oligonucleotides were synthesized with 6-Thio-dG to provide SH groups where TAMM can bind. C. A SDS-PAGE showing the gel retardation of the oligos once the TAMM has been bound to them. D. T30S subunits were incubated with an excess of ^{35}S-Cys-TAMM-DNA oligonucleotides, complementary to the 3′ end of the 16S rRNA. The reaction mix was placed onto a sucrose gradient, showing that the labeled oligonucleotides bind to T30S subunits.

teins with seleno-methionine. The selenated proteins may be reconstituted into cores lacking them. To develop a procedure for specific and quantitative protein detachments from the T30S, many reagents and various conditions have been examined [Table I]. Most of the reagents were found to have no effect on the integrity of the small ribosomal subunit. However it was found that ten proteins (TS2, TS3, TS5, TS9, TS10, TS12, TS13, TS14, TS18, and TS19) were detached from the T30S by high concentrations of $MgCl_2$ or LiCl. Interestingly, both salts

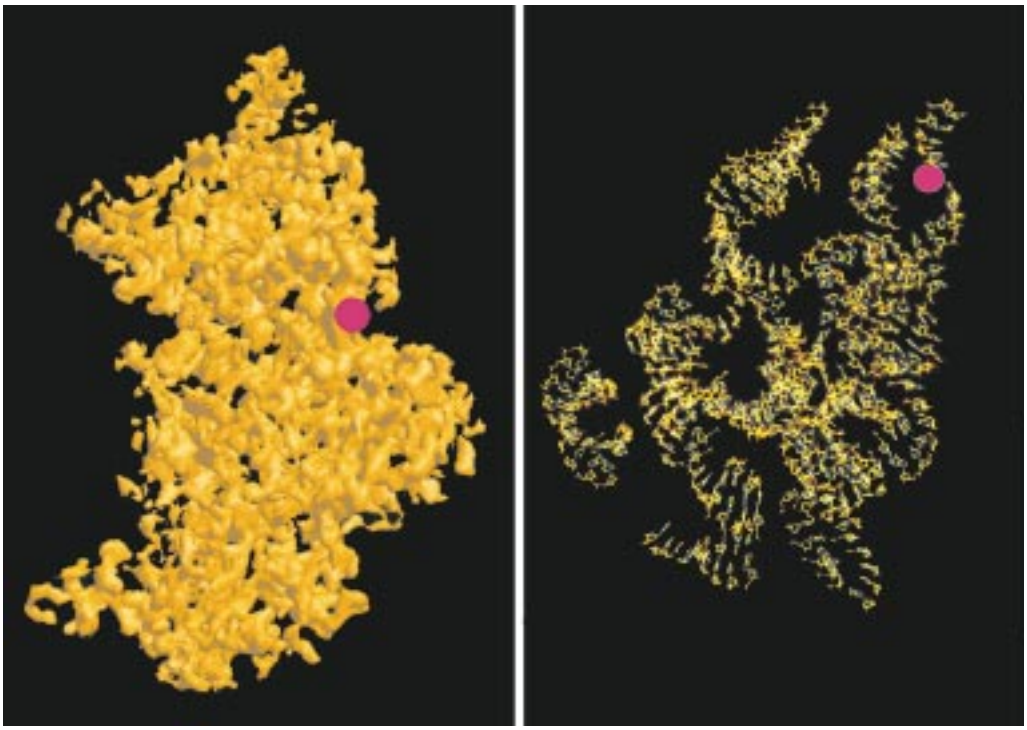

Figure 2: The locations of two TAMM molecules that were bound to the DNA oligomers complimenting the 3′ end, as seen in the 7.2 A map of T30S (26,27). The binding site is shown as two red balls.

led to the detachment of the same proteins. These were isolated by FPLC and identified by two-dimensional-PAGE. The detached proteins could be totally reconstituted into the ribosomal core particles even after FPLC runs.

Protein TS5:
Protein TS5 was chosen, because it splits totally from the small ribosomal subunit and it has five methionines that may be replaced by seleno-methionine. Furthermore, its structure as an isolated protein has been determined (14). The protein was cloned and overexpressed in its native form and as a seleno-methionine analogue. The overexpressed TS5 was purified and its identity was verified by amino-acid-analysis and by N-terminal analysis. The exchange of methionine by seleno-methionine was proven by mass-spectroscopy. Thus five anomalous scatterers were inserted into the protein.

The small ribosomal subunit contains about 20 methionines. The minimum number of selenium atoms that are supposed to yield phase information should be

Table I
The different conditions that were examined for their effect on the T30S.

#	Conditions	Notes
1	4 M LiCl, $H_{10}M_{10}N_{60}$	nine proteins completely detached, TS12 partially (see below)
2	4 M LiCl, $H_{10}M_{100}N_{60}$	same split as #1
3	2 M $MgCl_2$ in $H_{10}M_{10}N_{60}$	same split as #1
4	10% MeOH O/N then 4 M LiCl in $H_{10}M_{10}N_{60}$	same split as #1
5	25% methanol O/N then 4 M LiCl in $H_{10}M_{10}N_{60}$	same split as #1
6	50% methanol O/N then 4 M LiCl in $H_{10}M_{10}N_{60}$	same split as #1
7	7 mM $CoCl_2$ O/N, then 4 M LiCl in $H_{10}M_{10}N_{60}$	same split as #1
8	7 mM $NiCl_2$ O/N, then 4 M LiCl in $H_{10}M_{10}N_{60}$	same split as #1
9	14 mM $CoCl_2$ O/N, then 4 M LiCl in $H_{10}M_{10}N_{60}$	same split as #1
10	10% MeOH O/N then 3 M LiCl in $H_{10}M_{10}N_{60}$	more proteins detached then #1, but just partially
11	10% MeOH O/N then 3.5 M LiCl in $H_{10}M_{10}N_{60}$	more proteins detached then #1, but just partially
12	MES (5.5) $_{20}M_{0.3}N_{60}$	no effect on T30S
13	MES (5.5) $_{20}M_{0}N_{60}$	no effect on T30S
14	H $_{20}M_{0.3}N_{60}$	no effect on T30S
15	H $_{20}M_{0}N_{60}$	no effect on T30S
16	Imidazole (9.0) $_{20}M_{0.3}N_{60}$	no effect on T30S
17	Imidazole (9.0) $_{20}M_{0}N_{60}$	no effect on T30S
18	electroporation 2.5kV, 5″ in $H_{10}M_1N_{30}$	no effect on T30S
19	Glycine (9.5) N_{60} O/N, 4°C, D	no effect on T30S
20	Citrate (5.25) N_{60} O/N, 4°C, D	no effect on T30S
21	electric field, 5mA, 10' in $H_{10}M_1N_{30}$	no effect on T30S
22	1% CHAPS, O/N, 4°C, D in $H_{10}M_{10}N_{60}$	no effect on T30S
23	7 mM $CoCl_2$ O/N, 4°C, D in $H_{10}M_{10}N_{60}$	no effect on T30S
24	7 mM $NiCl_2$ O/N, 4°C, D in $H_{10}M_{10}N_{60}$	no effect on T30S
25	$H_{20}M_1N_{60}$, O/N, 4°C, D	no effect on T30S
26	$H_{10}M_{10}N_{60}$, 60°C, 6 hrs	no effect on T30S
27	$H_{10}N_{60}$, 60°C, 6 hrs	no effect on T30S
28	3 M NaCl in $H_{10}M_{10}N_{60}$	no effect on T30S
29	2.5 M NH_4Cl in $H_{10}M_{10}N_{60}$	no effect on T30S
30	7 mM $CuCl_2$ O/N, 4°C, D, in $H_{10}M_{10}N_{60}$	T30S denatured
31	5 mM cis-Platinum + 4 M LiCl O/N in $H_{10}M_{10}N_{60}$	T30S denatured
32	5 mM cis-Platinum derivative + 4 M LiCl O/N in $H_{10}M_{10}N_{60}$	T30S denatured
33	H_{10}, 60°C, 6 hrs	T30S denatured
34	$H_{10}N_{60}$, 60°C, 6 hrs	T30S denatured
35	10 mM Na_3BO_3 (9.0), 60°C, 6 hrs	T30S denatured
36	1 M $MgCl_2$ + 4 M LiCl	T30S denatured

Remarks: H_{10} = Hepes 10 mM (7.8), M_{10} = $MgCl_2$ 10 mM, N_{60} = NH_4Cl 60 mM, D = dialysis, O/N = overnight. Numbers in brackets indicate the pH of the buffer. The proteins that were split: TS2, TS3, TS5, TS9, TS10, TS12 (partially), TS13, TS14, TS18, TS19.

around 70 per one 30S subunit. Therefore we designed procedures to insert 3-10 seleno-methionine residues on each of the proteins which were split quantitatively, by genetic engineering. An example is TS5, which was modified by the addition of two tails, one on the N-terminus (5 or 3 methionines) and one on the C-terminus (3 methionines). Based on sequence analysis of *E.coli* ribosomal proteins, it was shown that the initial methionine is normally not removed if followed by certain amino acids (i.e. lysine or arginine). The N-terminus was mutated by adding the following amino acids met-lys-met-met-met-(met-met). This sequence was chosen to ensure that even if the initial methionine is cleaved the tail should remain intact.

Flags and Markers

Positioning of the ribosomal components in the electron density maps poses additional challenges as besides visual incorporation of the non-crystallographic structural information (e.g. electron microscopy, neutron scattering etc.) it requires independent crystallographic information. Obtaining this type of information is a rather complicated and time demanding approach, but it should lead to unbiased map interpretation. In a procedure which was developed by us, medium-size heavy atom markers (i.e.TAMM or a tetrairidium cluster (15)) are attached directly to selected sites on the ribosomal surface or via carriers with a high affinity for the ribosomal particles, either prior to or after crystallization. Examples for carriers are antibiotics, complementary DNA (cDNA) oligomers, tRNA molecules and factors participating in the translation process.

So far we have elucidated the sites of two specific cysteines. Labeling studies on the T30S subunit in solution showed one fully and one partially exposed -SH group belonging to proteins TS11 and TS13, respectively. These were used for covalent binding of a monofunctional reagent of the tetrairidium-cluster or of TAMM molecules prior to crystallization. The crystals obtained from the modified particles diffract to 4.5Å resolution (8) and are isomorphous with the native ones. The attachment of 1-2 equivalents of the tetrairidium cluster yielded a weak derivative, albeit a powerful marker. Thus, two prominent peaks were revealed in the electron density map using the amplitudes of the data that were collected from the tetrairidium modified crystals with the 7.2Å MIR phases of T30S (8).

The minor site, assigned as the cysteine of TS13, is located on the particle's "head" in a position similar to that assigned to S13 in *E. coli* 30S, according to immuno electron microscopy (16), modeling (17) and neutron scattering (18). The major site, assigned as cystein of TS11 is located at the central part of the particle, in a position roughly compatible with that suggested by immuno electron microscopy for protein S11 in *E. coli* 30S, as well as by modeling the ribosomal components within the envelope of cryo-EM reconstructions. However, it deviates by approximately 35Å from the position assigned to the center of mass of this protein by studies exploiting neutron scattering and contrast variation. Since the TAMM, the tetrairidium cluster and the immuno electron microscopy target the surface of the ribosomal particles, whereas the neutron scattering triangulation approximates the positions of the centers of mass of the ribosomal proteins, this deviation is tolerable.

Hybridization with cDNA

It was previously shown that some regions of the rRNA are accessible to hybridization with short cDNA oligonucleotides (19-25). We synthesized over a dozen oligonucleotides complementary to specific regions of the 16S rRNA. These contained at least one 6-thio-deoxyguanosine, to which heavy atoms were bound covalently. The length of the oligomers (16-20 bases) was designed so that the expected hybrid double helix has maximum stability. Only cDNA oligomers that were found to hybridize in solution in close to stoichiometric ratios were used in this study. The TAMM-labeled-oligonucleotides were used for crystal soaking.

Some of the non-modified, as well as the heavy atom modified oligonucleotides were found to have high affinity for their complementary sequence in the 16S rRNA. However, co-crystallization of the 30S subunits with cDNA fragments was not always successful. As hybridization is an equilibrium process, the hybrids may partially dissociate during the long time needed for crystallization. In this respect, diffusion of the cDNA fragments into the crystals was found more suitable. The latter is a rather short process that can be promptly terminated by the shock freezing needed for data collection, once it is assumed that the hybridization of the cDNA to RNA is completed. Clearly, the available volume within the crystal for the movement of the DNA fragment is rather limited. In fact, such long heavy atom carriers (up to 70Å) are not commonly used in protein crystallography. It is conceivable that the large continuous solvent regions within the crystals allow for their diffusion.

In order to check the influence of the oligonucleotide hybridization on the crystal properties, initially the non-modified oligonucleotides were diffused into native T30S crystals. It was observed that the oligonucleotides have a marked effect on the resolution of the crystals. Some of them led to a decrease in resolution, probably because they bind to regions involved in the crystal network, or because their binding induced conformational changes. Others did not alter the resolution of the crystals. Some even seemed to improve the crystal's internal order, probably by stabilizing certain flexible regions. The highest resolution was obtained when a 22mer oligonucleotide complementary to the 3′ end bases of the 16S rRNA was used for soaking. Crystals soaked in this modified oligonucleotide gave isomorphous derivatives diffracting to 3.4Å. Preliminary crystallographic analysis was performed by constructing a difference Fourier map at 7.2Å resolution. This map showed peaks, which should account for the TAMM molecules bound to the cDNA tail (Figure 2). In contrast, no data could be collected from crystals to which long DNA oligomers complementing the 16S rRNA around base 1400 were diffused, because of a marked resolution drop from 3Å to 12-15Å which was accompanied by a dramatic increase in the mosaic spread.

The derivatization by the modified DNA oligomers should be carefully performed, since not only hybridization but also additional, undesired interactions may form. Hence, special effort is being made to limit the chemical reactivity of the heavy atoms that are bound to the DNA oligomers, in order to direct them only to the desired positions.

Conclusions and Future Plans

We have shown that experimental phases yielded a medium resolution electron density map for the small ribosomal subunit of *Thermus thermophilus* which exhibits the external shape of this subunit at a close to its functional conformation. This medium resolution map contains recognizable features and a significant part of it was interpreted at a level close to molecular resolution. In this studies we extended the level of map interpretation. Thus, selected locations were revealed by covalently bound heavy atom compounds or by materials that have a high affinity to accessible regions of the ribosome, to which heavy atoms had been bound.

A wealth of structural information has been extracted from the current map. Of particular interest is the high versatility in the interactions between the ribosomal components. The quality of the current map indicates that more interesting features should be revealed once higher resolution data are available. At that stage, studies of the functional-dynamic aspects of the process of protein biosynthesis may also be addressed. Thus, we may provide tools for monitoring various functions by exploiting tailor made ligands, such as heavy metal modified antibiotics or complementary DNA to exposed single strand rRNA regions. The studies reported here have been designed for achieving these goals.

We also showed that the ribosomal protein TS5 can be modified to include seleno methionine. Employing this procedure to other ribosomal proteins that can be detached reversibly from the T30S particle, the number of inserted selenium atoms may be sufficient for MAD phasing of the diffraction of the entire T30S particle.

An approach for exploiting the cDNA oligomers as heavy atom carriers was designed, using compounds containing several Hg atoms bound at several positions. This can be achieved in two ways: (a) binding methyl-mercury acetate to the thiolated guanosines mentioned above; and (b) direct mercuration of the cytosine and uracil bases with mercuric acetate. For the latter we replaced the DNA thymines by uracils, since the reagent reacts almost exclusively with cytidines and uridines (8). In this procedure the oligomers were extensively mercurated, as indicated by PAGE of their ^{32}P phosphorylated derivatives. This work is still in progress.

Abbreviations

70S, 50S, 30S: the whole ribosome and its two subunits from prokaryotes, respectively. A letter prefix to the ribosomal particles or ribosomal proteins represents the bacterial source (E=*Escherichia coli*; T=*Thermus thermophilus*). The names of the ribosomal proteins are composed of a prefix showing the bacterial source, the letters L or S; showing that this protein is of the large or small subunit, and a running number; according to sequence homology to *E. coli* ribosomal proteins; MAD = multiple anomalous dispersion; MIR = multiple isomorphous replacement; PAGE = Polyacrylamid-gel-electrophoresis; SDS = sodium- dodecyl-sulfate; TAMM = tetrakis(acetoxymercuri)-methane CHAPS = (3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate), ; IPTG = Isopopyl β-D-Thiogalactopyranoside, O/N = overnight

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Appendix:

$H_{10}M_{10}N_{60}$:
- 0.01 M Hepes buffer pH = 7.8 (NaOH)
- 0.01 M MgCl2
- 0.06 M NH4Cl

Lysis Buffer:
- 10 mM Tris-HCl pH 8.0
- 5 mM EDTA
- 100 mM NaCl
- 0.5% SDS
- +75 mg Proteinase K to 10 ml

Resuspension buffer 0.8 M NaCl:
- 20 mM MOPS pH 7.5

• 100 mM NH_4Cl
• 10 mM $MgCl_2$
• 7 mM mercaptoethanol
• 0.1 mM benzamidine
• 0.1 mM PMSF
• 0.8 M NaCl

Reconstitution buffer:
• 20 mM Tris 7.5
• 20 mM $MgCl_2$
• 0.2 mM EDTA
• 400 mM NH_4Cl
• 4mM mercaptoethanol

Dialysis buffer F (pH 7.5):
• 20 mM MOPS
• 7 mM b-mercaptoethanol
• 0.1 mM benzamidine
• 0.1 mM PMSF
• 50 mM NaCl

The bacterial growth media are described in (28).

Reference and Footnotes

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