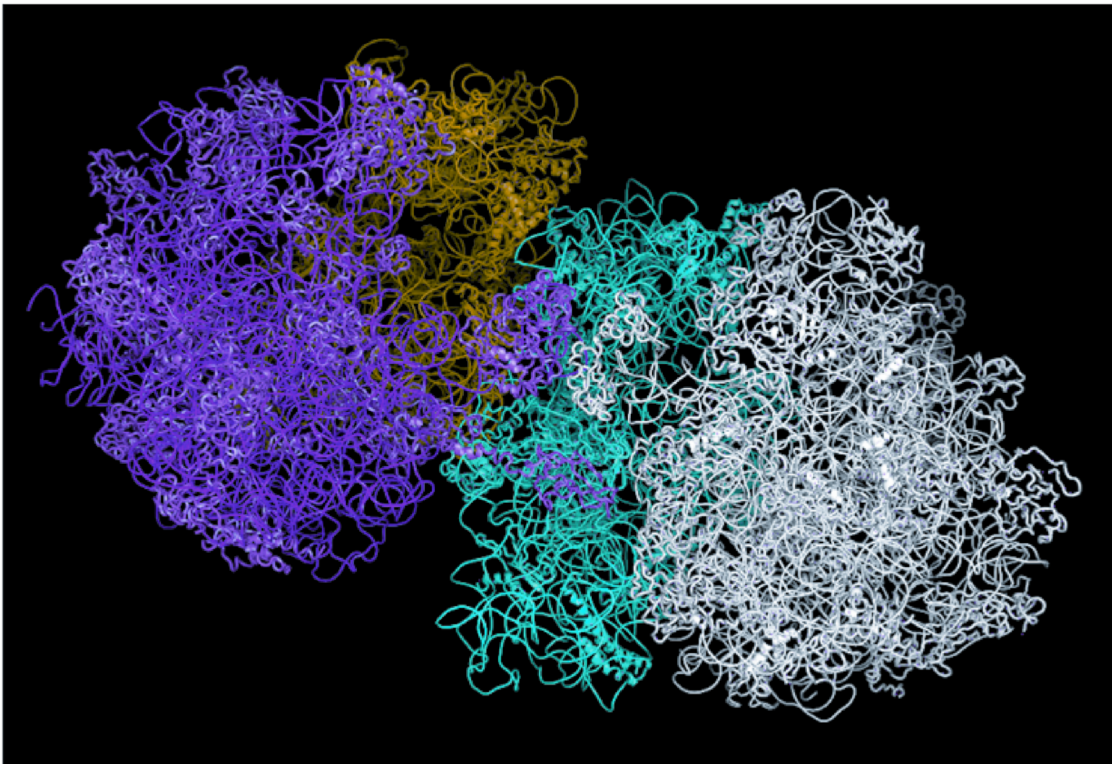
Towards atomic resolution structures of the *E. coli* 70S ribosome



Chapter II

Abstract

The structures of the *E. coli* 70S ribosome has been solved by x-ray crystallography up to 3.5 Å resolution**.** The new crystal form was found to contain two ribosomes per asymmetric unit that have two different conformations that may reflect two stages of the ribosome within the elongation cycle. In addition, the new crystal form has been shown to be suitable for soaking experiments, by using the antibiotic kasugamycin and the Ribosome Recycling Factor (RRF) from *E. coli* and *T. thermophilus*. Difference electron density maps show kasugamycin bound to the ribosome at a resolution of 3.5 Å. Electron density attributed to domain I of RRF can be seen to 8Å in the case of *E. coli* and to 4.5Å in the case of *T. thermophilus*.

The 3.5 Å structure of the empty E. coli ribosome, and the observation that the new crystal form is suitable for soaking experiments can be seen as the starting point for obtaining structures of different ribosome complexes of the translation cycle. These structures should provide a significant advancement towards an understanding of the molecular mechanisms involved in protein synthesis.

Introduction

Since the determination of the atomic resolution structures of two ribosomal subunits 30S (Wimberly, Brodersen et al. 2000), (Schluenzen, Tocilj et al. 2000), (Carter, Jr et al. 2000), (Brodersen, Clemons et al. 2000), (Ogle, Brodersen et al. 2001), (Carter, Clemons et al. 2001), (Ogle, Murphy et al. 2002), (Murphy, Ramakrishnan et al. 2004) and 50S (Ban, Nissen et al. 2000), (Nissen, Hansen et al. 2000), (Harms, Schluenzen et al. 2001), (Bashan, Agmon et al. 2001), (Hansen, Ippolito et al. 2002) as well as the mid-resolution structures of the complete 70S ribosome (Cate, Yusupov et al. 1999), (Yusupov, Yusupova et al. 2001), (Yusupova, Yusupov et al. 2001) the goal was to obtain crystallographic data of the 70S ribosome in different states of protein synthesis at atomic resolution. This together with the numerous biochemical studies performed in the last few decades would finally lead to a complete picture of the mechanism of translation in molecular detail.

1. Crystallizing the *E. coli* 70S ribosome

Over 40 years of genetic, biochemical and structural research has been

performed on the 70S ribosome of the eubacterium *Escherichia coli* (*E. coli*), making it the ribosome that has been best genetically and biochemically characterized. It thus seemed reasonable that a structure of the *E. coli* ribosome would be of significant help in understanding the mechanistic details of protein synthesis that remained unanswered from biochemical experiments. The structure would also have enormous medical relevance since the prokaryotic ribosome is one of the main targets of antibiotics. Understanding the ribosome in molecular detail would help in the design of new drugs that are necessary due to increasing antibiotic resistance of bacteria.

Although the first crystals of the *E. coli* ribosome were reported in 1982 (Wittmann, Mussig et al. 1982) researchers seemed to have more success in crystallizing and solving ribosome structures of extremophile ribosomes (see General introduction of this thesis). However, in 2003 the laboratory of Jamie H. D. Cate solved the first structure of the *E. coli* ribosome in 2003 to 8.7Å resolution (Vila-Sanjurjo, Ridgeway et al. 2003).

2. The content of protein S1 in crystal structures

The 30S protein S1 is the largest ribosomal, consisting of 557 amino acids posses a molecular weight of 68kDa (Wittmann 1974). S1 has been proposed to be necessary in some cases in translational initiation (Tzareva, Makhno et al. 1994) and elongation (Potapov and Subramanian 1992). Additionally, it was found to have a high affinity for mRNA (Draper and von Hippel 1978). In a recent

cryo-Electron-Microscopy (EM) structure published by Joachim Frank’s laboratory, the location of S1 on the 30S subunit was shown for the first time (Sengupta, Agrawal et al. 2001). Due to its weak and reversible association to the 30S subunit S1 had never been visualized in a crystal structure. In fact, researchers have intentionally removed the protein in order to obtain better diffracting crystals (Wimberly, Brodersen et al. 2000).

3. The ratchet-linker model (Frank and Agrawal 2000)

Already in 1968 a model for the translocation step was postulated which involved a relative movement of the two ribosomal subunits (Bretscher 1968).However, the relative movement of the two ribosomal subunits has been inferred by recent cryo-EM structures of Joachim Frank’s laboratory (Frank and Agrawal 2000), (Valle, Zavialov et al. 2003). These studies revealed a 19 Å ratchet-like movement of the 30S subunit with respect to the 50S subunit upon binding of EF-G in complex with the non-cleavable GTP analog GMPPNP (**FIGURE 20**). The 30S subunit was found to rotate back after GTP hydrolysis. This observation was further investigated by a second cryo-EM study from the same group (Valle, Zavialov et al. 2003) and strengthened by biochemical results performed by Ehrenberg and co-workers (Zavialov and Ehrenberg 2003). It was revealed that a deacylated tRNA at the P site is required for this movement to occur. In the presence of a peptidyl tRNA at the P site, the ribosome is ‘locked’ in a conformation that prohibits the rotation of the 30S subunit. After peptide bond formation, resulting in a deacylated tRNA at the P site and a peptidyl tRNA at the A site, the ribosome switches to an ‘unlocked’ conformation. The

‘unlocked’ conformation allows EF-G/GTP to bind resulting in the pre-translocational conformation. EF-G/GTP then translocats the A- and P site tRNAs to the P and E site respectively. This promotes the movement of the 30S, which is accompanied by a movement of the L1 stalk towards the E site resulting in the post-translocational complex. The simultaneous movement of the 30S and the L1 arm is reversed by EF-G hydrolysis.

4. Kasugamycin

Antibiotics have been heavily used in the ribosome field. The reasons for this are not only medical, since the prokaryotic ribosome is the main target for antibiotics, but also scientific as these drugs are very helpful in understanding the mechanism of protein synthesis.

The aminoglycoside kasugamycin (ksg) (see **Figure 34A** for its structure) is commonly used in the treatment of rice fields against the fungus *Piricularia oryzae* (Umezawa, Hamada et al. 1965). The drug has been used clinically in the treatment against infections due to *Pseudomonas aeruginosa* (Ishigami, Fukuda et al. 1967). Ksg was found to inhibit translation initiation (Okuyama, Machiyama et al. 1971), (Okuyama and Tanaka 1972), (Tai, Wallace et al. 1973) by blocking the binding of the initiator tRNA (fMet $tRNA^{fMet}$) to the ribosome (Okuyama, Machiyama et al. 1971), (Poldermans, Goosen et al. 1979). By means of chemical probing, kasugamycin was found to alter the reactivity towards chemical probes of three different residues of the 16S rRNA. It enhances the reactivity of C795 and protects the tRNA diagnostic sites A794 and G926 (Woodcock, Moazed et al. 1991), (Moazed and Noller 1989). In addition,

kasugamycin resistance mutations, A794G, G926A and A1519C have been isolated by means of genetic screens (Vila-Sanjurjo, Squires et al. 1999). The x-ray structure of the aminoglycoside, kasugamycin (ksg) in complex with the ribosome or with the 30S subunit has never been solved.

5. Ribosome Recycling Factor (RRF)

RRF, with a molecular weight of ~ 21,000 Dalton is a heat-shock protein (Teixeira-Gomes, Cloeckaert et al. 2000) that was found to be essential in prokaryotes (Janosi, Shimizu et al. 1994) and eukaryotes (Rolland, Janosi et al. 1999), (Teyssier, Hirokawa et al. 2003).

As shown by atomic resolution structures of four different species (**Figure 21**) ((Selmer, Al-Karadaghi et al. 1999), (Kim, Min et al. 2000), (Yoshida, Uchiyama et al. 2001), (Toyoda, Tin et al. 2000), (Nakano, Uchiyama et al. 2002) & (Nakano, Yoshida et al. 2003)) RRF consists of two domains: domain I comprised of a 3 helix bundle, and domain II consisting of six _-sheets and two _-helices. The two domains are connected through a flexible hinge which may explanain why the orientation of the two domains in the four crystal structures is different (**Figure 21A**). Strikingly, the overall shape of RRF is very similar to the one of a tRNA (superposition of RRF and tRNA can be seen in **Figure 21B**) prompting the proposal of a structural and functional mimicry between the two molecules (Selmer, Al-Karadaghi et al. 1999). Selmer et al proposed that RRF would bind to the A site from which it would be translocated with the help of EF-

G/GTP to the P site, resulting in release of the deacylated tRNA from the E site and the dissociation of the subunits. The mimicry hypothesis was dismissed by hydroxyl-radical (Lancaster, Kiel et al. 2002) and cryo-EM (Agrawal, Sharma et al. 2004) analysis showing that the factor binds in a fashion that is completely form that of a tRNA. However, the fact that RRF and EF-G have overlapping binding sites (Agrawal, Sharma et al. 2004) and the fact that RRF is moved by EF-G prior to mRNA release suggests a similarity between RRF and tRNA movement on the ribosome.

6. The x-ray structures presented here

A new crystal form of the *E. coli* 70S ribosome was obtained that diffracts x-rays to a resolution of 3Å. The collection, solving and refinement of a 3.5Å dataset which provides the first near atomic resolution structure of the entire *E. coli* 70S ribosome is discussed in this part. This structure can be seen as the first snapshot of an atomic resolution 'movie' which is the ultimate goal of our laboratory and other researchers in the ribosome field. In addition, it could be shown that the new crystal form is suitable for performing soaking experiments, suggesting that crystal structures of ribosome complexes may be solved in the future.

The antibiotic kasugamycin was soaked into the new crystal form revealing the binding position of the drug between the P - and E site of the 30S subunit where it is known to inhibit translation initiation. In addition, the Ribosome