Recycling Factor (RRF) from two different species (*E. coli* and *T. thermophilus*) was soaked into the new crystal form, showing that soaking experiments are also feasible with the new crystal form.

Results

1. Crystallization of the E. coli 70S ribosome

Obtaining high resolution crystals of the 70S ribosome as well as the process of data collection was accompanied by a lot of difficulties which will be described in these paragraphs.

1.1 S1 content

Initially, 70S ribosomes were prepared as tight – coupled 70S (70S-T) as described in (Vila-Sanjurjo, Ridgeway et al. 2003) and in the Material and Method section of Chapter I. The crystals obtained were dissolved and their content analyzed on a composite gel (Dahlberg 1979). The gel separates three different 50S forms (50S, large subunit containing all the proteins, 50S-M, large subunit containing all the proteins but L7/L12, 50S-F, large subunit containing all the proteins but L7/L12, L11 and L10) and two different 30S forms (30S, small subunit containing all the proteins and 30S-F, small subunit containing all the proteins but S1) shown in **Figure 22C**. The gel revealed that most of the crystals did contain 70S ribosomes missing protein S1 (date not shown), suggesting that the removal of the protein might result in even better crystals. We attempted to

completely remove protein S1 during the 70S ribosome preparation by dissociating the subunits and treating them with Poly-U mRNA, known to strip of S1 (Draper and von Hippel 1978), before re-association. **Figure 22A** shows the Poly-U treated subunits run on a sucrose gradient resulting in a Poly – U mRNA - S1 peak and a 30S and 50S peak. The Poly-U – mRNA - S1 peak was used as a S1 marker to examine the S1 content of the re-associated 70S (70S-R) (see **Figure 22B**). Composite gel analysis (Grabowski and Dahlberg 1990) of 70S-R revealed that S1 was completely removed (**Figure 22D**, lane 2 and 3), in contrast to the 70S-T preparation that only contained about 50% S1 depleted 30S (**Figure 22D**, lane1).

Removing of protein S1 was one attempt that improved the quality as well as the quantity of crystals diffracting between 3Å and 3.5Å.

1.2 Temperature gradient + pH screen

Crystals were obtained initially obtained using the vapor diffusion technique in the presence of PEG (Polyethyleneglycol) as a precipitant. They were then optimized using the batch crystallization technique in the presence of a 6-12% MPD and 2-4% PEG mixture at pH 6. By applying a temperature gradient, ranging from 4°C - 25°C - 4° within 24 hours (using the M6 Incubator, Hampton Research), before letting the crystals grow at 4°C the appearance of showers of small crystals could be prevented.

A change of the pH from 6 to 7.5 resulted in high quantities of reproducible, long and chunky (100 µm x 100 µm x 300-600 µm) crystals (see

Figure 23) appearing after 4 - 6 weeks of growing at 4°C that would diffract between 3Å and 3.5Å. The orthorombic crystals, spacegroup $P2_12_12_1$ (a = 208.9, b = 379.4, c = 739.4, α, β, γ = 90°) contain two 2.5 x 10^6 Dalton 70S ribosomes per asymmetric unit, with a solvent content of 56%.

1.3 Mounting of crystals

Crystals were initially stabilized by adding 100% of the cryo - protecting reagent directly to the crystal well followed by an overnight incubation at 4°C. Since this resulted in cracking of some crystals, the stabilization procedure was improved by adding the cryo-coolant reagent to the well solution step - wise over a period of 36 hours. Crystals were mounted so that most of the crystal would stick out of the loop (**Figure 24A**). This method allowed us to direct the beam to the untouched part of the crystal which consistently yielded a better diffraction pattern than the part within the loop.

1.4 Crystals in the beam

For a crystal to diffract in all three dimensions to the same resolution they had to have a chunky shape, in which the two shorter dimensions (axes a and b) should be about 100μm in each direction. The longest dimension of the crystal corresponds to the c axis. When the crystal was oriented with this axis parallel to the x-ray beam, diffraction up to 3Å required up to 420 seconds exposure time.

Obtaining the same resolution when the crystal was oriented with either the a and b axis parallel to the beam, could be achieved by using an exposure time of 90-150 seconds. Crystals were stable in the beam, lasting up to 24 hours.

The first 60 % of the complete dataset to 3.5 Å resolution was collected using an oscillation range of 0.3 ° resulting in mosaic spread between 0.1° and 0.35°. Due to overlap problems, the last 34 % of the dataset were collected using an oscillation range between 0.2° and 0.1°. Additionally, increasing the distance of the detector to the crystal helped to avoid overlaps. The use of bendable loops (Hampton Research, **Figure 24C**) instead of non-bendable ones (**Figure 24B**) allowed one to get the c axes of the crystals parallel to the beam obtaining the missing data. Due to the beamline-setup the loops had to be bent in a certain way to allow a 360° rotation of the crystal (**Figure 24C**).

In order to optimize the collection strategy, we used a program written by James Holton (beamline scientist at beamline 8.3.1 at the Advanced Light Source) that reads in the reflections already collected (4 1994) and calculates the missing reflections and determines the optimal position of the crystal in degrees (phi) to complete the dataset (**Figure 24D**).

1.5 Icerings

Many of our crystals were surrounded by a fine layer of ice resulting in ice rings on the diffraction pattern between 3.6 and 3.9 Å (see **Figure 25A**). Although it was possible to clean off the ice from most crystals (see **Figure 25B)** by washing with liquid nitrogen or brushing with a piece of kimwipe, the icerings

became a problem during data processing, as shown by the high chi2 values for the resolution bins containing the ice rings (3.9Å and 3.6 - 3.7Å) after merging the datasets with SCALEPACK (Otwinowski 1991). In order to lower the chi2 values in these regions, the data was reprocessed in DENZO using the command line "reject slope 20 (set by default to 50). **Figure 25C** shows the output of the final scalepack file after several rounds of rejection that was then used for the calculation of electron density maps.

2. Building the model & Refinement

2.1 Molecular replacement

The phase problem was solved by molecular replacement using the program 'Phaser' (Storoni, McCoy et al. 2004)**.** The atomic resolution structure of the *Deinococcus radiodurans* 50S subunit (Harms, Schluenzen et al. 2001) and the *Thermus thermophilus* 30S subunit (Wimberly, Brodersen et al. 2000) were used to build an initial model for one 70S ribosome that was refined at 9 Å resolution using rigid-body refinement (Vila-Sanjurjo, Ridgeway et al. 2003). The resulting model was used as the search model in 'Phaser'. The new crystal form contains two ribosomes per asymmetric unit whose position 'Phaser' was able to predict. The positions were then used in rigid body refinement (CNS program (Brünger, Adams et al. 1998)) to verify that the solution could be used to solve

the phase problem.

2.2 Bulding the model

The model still contained the sequence of *Deinococcus radiodurans* (50S) and *Thermus thermophilus* (30S) and had to be replaced with the *E. coli* sequence. Since 2/3 of the ribosome consists of RNA and only 1/3 of protein, swapping the RNA sequences should have a bigger impact on the R_{free} than the proteins would. In order to swap the sequences we used the secondary structure maps of 16S rRNA and 23S rRNA to locate homologous structural regions for which we could directly do the replacement (**Figure 26**) (Gutell). A program written by Wen Zhang in Jamie HD Cate's laboratory was very helpful for swapping the sequences between two different species in regions that did not contain any insertions or deletions.

The program changes the starting sequence (*Deinoccocus radiodurans* for 23S rRNA and *Thermus thermophilus* for 16S rRNA) into the final sequence (*E. coli*) and writes it into a PDB-formatted coordinate file that can be read into O (Jones, Zou et al. 1991). Final adjustments could be made in O (Jones, Zou et al. 1991) by fitting the *E. coli* model into the observed density. The structure of the 50S from *Haloarcula marismortui* turned out to be very helpful in the process of building the *E. coli* model since certain regions are more conserved between *E. coli* and *H. marismortui* (**Figure 26A and 26C, green arrows**) than *E. coli* and *D. radiodurans*.

In addition to directly replacing the sequence, one also had to deal with the presence of insertions and deletions**. Figure 26** shows an example of an insertion (red arrow), a helix within the 5' domain of 23S rRNA, which in E. coli

(**Figure 26A**) is far more extended than in *D. radiodurans* **(Figure 26B).** In this case, one had to rebuild the helix in the program O (Jones, Zou et al. 1991).

While building the model in O (Jones, Zou et al. 1991) it was important to make sure that the base pairs remained planar and that the sugars were in the right puckering mode. Special attention was required on bond lengths, bond angles and torsional angels (**Figure 27B**).

The region of the 5S rRNA within the 50S subunit was replaced with an atomic resolution structure of a fragement of 5S rRNA bound to protein L25 from *E .coli* from the T.A. Steitz laboratory (Lu and Steitz 2000).

Once the building of the rRNA is finished we will proceed to do likewise with the protein component of the ribosome. In fact, changing the protein sequence to that of *E. coli* is already in progress. S21, a protein of the 30S subunit in *E .coli* has to be rebuilt from scratch, since it does not exists in *Thermus thermophilus.*

2.3 Refinement

2.3.1 Torsional Dynamics

Refinement was performed with the help of the CNS program (Crystallography and NMR System) (Brünger, Adams et al. 1998). Base-pair restrains were applied by using the Saenger-Classification (**Figure 27A**) (Saenger 1983) as a reference. This method ensured that the distance of the hydrogen bonds between two members of a base pair were reasonable (~ 2.8 Å between N and O atoms). The distances were enforced using the NOE formalism

within CNS. For all major base pair types (Watson-Crick, G-U wobble, and sheard G-A), the bases of the two nucleotides were also restrained to be planar with respect to each other. The simulated annealing method with torsion angle dynamics implanted in the CNS 1.1 program (Brünger, Adams et al. 1998) was used, with these constraints, to improve the R_{free}. **Figure 27B** shows the six different torsion angles that occur in ribonucleic acid: these are the angles α between P and O5', β between O5' and C5', γ between C5' and C4', δ between C4' and C3', ε between C3' and O3', ζ between O3' and P of the next residue.

2.3.2 Non Crystallographic Symmetry Averaging

One asymmetric unit contained two ribosomes that were in two strikingly different conformations. The conformational changes movements of the head and the spur region of the 30S subunit as well as the L1 and L7/L12 region of the 50S which are different in the two 70S molecules of the asymmetric unit. Non-crystallographic symmetry averaging was used for the parts of the ribosome that were in the same conformation, thus improving the R_{free} values and the density maps slightly.

In addition density modification was applied during the refinement process resulting also in an improvement of the electron density maps (Abrahams and Leslie 1996). This method determines the boundaries of a molecule and multiplies everything outside these boundaries (the solvent site) by a correction term before new phases are being determined for the new density modified map.

The crystallographic statistics are shown in **Table 3**. Since the refinement

as well as the complete conversion of the model to *E. coli* sequences are not yet completed the statistics are expected to improve.

2.4 Quality of the model

In order to show the quality of the model, B-factors diagrams for the rRNA of the 30S and the 50S subunit were created. **Figure 28A and 28B** show the B-factors of the 16S rRNA shown from the intersubunit space and the solvent site, respectively. Judging from the B-factors the model is already very good in the center of the small subunit where the blue and white regions, representing low B-factors (0-100 $Å^2$), are dominant. On the contrary, the outside of the 30S contains high (100-150$Å^2$, in **Figure 28** shown in red) B-factors, especially in the regions of the spur and the head, which are known to be flexible. Due to the flexibility of these regions the B-factors may always be a little bit higher than in the central regions even after further improvement of the model.

The B-factors for the 23S rRNA and 5S rRNA are shown in **Figure 29, Figure 29A** as viewed from the intersubunit space and in **Figure 29B** as viewed from the solvent site. As in the case of the 30S subunit, the B-factors for the outer regions of the 50S subunit are much higher than the ones for the inner regions. Again, the outer regions are known to be flexible, partly explaining the high B-factors. In addition, one has to mention that the region of the L1 and the L7/L12 arm are disordered in the structure, a phenomenon that was already seen in the atomic resolution structures of the 50S subunit from the laboratories of T.A.

Steitz (Ban, Nissen et al. 2000) and A. Yonath (Harms, Schluenzen et al. 2001).

2.5 Quality of electron density maps

Figure 30A-C show examples of different regions of the model that fit well into the $3F_{obs}$-2 F_{calc} electron density map (green). Even at only 3.5 Å resolution, it is possible to distinguish between pyrimidines and purines. Surprisingly, at the given resolution it is even possible to obtain in the F_{obs} - F_{calc} map positive electron density for hydrated magnesium ions (**Figure 30D**) that appear nicely coordinated by phosphate groups (shown in red).

3. Relative movement of the two subunits

As already mentioned above, one asymmetric unit contains two ribosomes. The position of the two ribosomes within the asymmetric unit is demonstrated in **Figure 19** (cover page of Chapter II, page 90) showing only the rRNA. The 30S of the first 70S molecule is shown in yellow and the corresponding 50S is shown in purple. The second molecule consists of a cyan 30S and a white 50S. Strikingly, the two molecules have two different conformations as illustrated in **Figure 31 and Figure 32** in which the 30S and 50S of the first molecule are shown in magenta (from now on called the 70S #1) and the ones of the second molecule are shown in yellow (from now on called the 70S #2).

3.1 Relative movement of the 30S subunit

Figure 31 shows the superposition of the two 30S subunits, with an rmsd (root mean square deviation) value of 1.9, looking onto the inter-subunit space from the top. A movement of 8 - 11Å is apparent in the head (H) region, increasing to 13Å in the beak (B) and spur (S) region. The head and beak region swing sidewise (following a half circle) towards the 50S subunit as indicated by the yellow arrow in the figure. The head and beak of the 70S #2's small subunit (yellow) leans further towards the ribosomal E site, following the direction of the mRNA movement. This conformational change is accompanied by an upwards movement of the spur region as indicated by the yellow arrow at the bottom of the **Figure 31**.

3.2 Relative movement of the 50S subunit

Super position of the rRNA of the two 50S subunits (**Figure 32)** (rmsd value of 1.3)**,** shows a conformational change of nearly 8 Å of the L1 arm and one ranging from 5 to 18 Å of the L7/L12 stalk.

3.3 Relative movement of the subunits in the context of the 70S ribosome

For the purpose of illustrating the conformational changes within the whole 70S ribosome, the subunits of 70S #1 and 70S #2 were superimposed. **Figure 33** compares the positions of the 30S and 50S subunit of 70S #1 (**Figure 33A**) to those of the subunits in 70S #2 (**Figure 33B**). The 30S subunit is colored in

yellow and the 50S subunit in magenta. The position of the subunits of the 70S #1 (**Figure 33A**) was taken as a reference to describe the conformational changes occurring within 70S #2 (**Figure 33B**). In the case of 70S #2 (**Figure 33B**) the head of the 30S subunit is leaning over towards the E-site (indicated by the yellow arrow), thereby opening up the mRNA entrance channel (Frank, Zhu et al. 1995), (Shatsky, Bakin et al. 1991). This leads to an enhanced access of the ribosomal A site. At the same time, the spur region is moving upwards (indicated by an upwards yellow arrow). There's no movement observed within the body of the small subunit.

The conformational changes of the 30S subunit are accompanied by a movement of the L1 arm of the 50S subunit away from the central protuberance (CP) and a shift of the L7/L12 stalk towards the 30S subunit. There is no movement of the L1 arm occurring towards the E site as seen in different X-ray and cryo-EM structures (Harms, Schluenzen et al. 2001), (Yusupov, Yusupova et al. 2001), (Gomez-Lorenzo, Spahn et al. 2000), (Valle, Zavialov et al. 2003). As in the case of the 30S subunit, no movement within the body of the 50S subunit has been observed in this structure.

A similar movement to that occurring in 70S #2 is proposed in the ratchet-linker model derived from cryo-EM analysis (Frank and Agrawal 2000). The similarities and differences between our observations and the ratchet-linker model are discussed in detail below.

4. Soaking experiments

To test whether the new crystal form could be used to solve the structure of biologically relevant complexes, we perfomed soaking experiments using the antibiotic kasugamycin and the ribosome recycling factor (RRF).

4.1 Kasugamycin (ksg)

The aminoglycoside kasugamycin (**Figure 34A**) was soaked into the new crystal form and a 60% complete dataset up to 3.5Å was collected (for crystallographic statistics see **Table 3**). An F_{obs} - F_{obs} electron difference map of the unliganded versus the ligand bound ribosomes reveals positive density for ksg on the two copies of the 30S subunit contained in the asymetric unit (**Figure 34B, C, and D**). Interestingly, the density lies between residues G926 and A794 of the 16S rRNA. This result is in agreement with chemical probing studies showing that the reactivity of these two sites is protected by the binding of the drug (Woodcock, Moazed et al. 1991) and by mutational analysis indicating that the same residues are sites of resistance to the antibiotic (Vila-Sanjurjo, Squires et al. 1999). Interestingly, the density overlaps the path of the mRNA in the E and P site codons (**Figure 36**). The significance of this will be discussed below.

4.2 Ribosome Recycling Factor (RRF)

T. thermophilus RRF (ttRRF) and *E .coli* RRF (ecRRF) were soaked into the new crystal form. As a result, we calculated preliminary difference electron

density maps for both factors to 8Å resolution. This allowed us to determine wether the factors were bound or not. Next, a 4.5 Å dataset for the crystals in complex with RRF from *T. thermophilus* was collected to ~60% completeness.

All three $F_{obs} - F_{obs}$ difference electron density maps from ecRRF (8 Å) and ttRRF (8Å and 4.5Å) show positive density in the intersubunit-cavity of the 50S subunit (**Figure 35A, B**) that can be attributed to domain I of the RRF when compared to the map of the empty 70S ribosome. In neither case, density for the second domain of the factor is observed, suggesting that it is disordered. This is in agreement with the observation that domain I forms a more stable interaction with the ribosome compared to domain II, suggesting that it functions as the main ribosome-anchoring site (Agrawal, Sharma et al. 2004).

Strikingly, regardless of the factor used in the soaks, positive density can only be seen in one of the ribosomes in the asymmetric unit. The functional implications of this observation will be discussed below.

Since RRFs from two different species were used it was tempting to investigate if the two would bind to the ribosome in the same way. Superposition of the electron density maps obtained with the two factors revealed that they bind in the same region, the A and P site of the 50S subunit. The tip region of domain I binds near G2253 (**Figure 35C**), known to interact with the CCA-end of the peptidyl tRNA in the P site (Moazed and Noller 1989). In addition RRF is in close proximity of residues U1946 and C1947 of helix 71 (**Figure 35C**), which is part of an inter-subunit bridge (Cate, Yusupov et al. 1999). The factor binds near to pseudo-uridine 1915 and A1916 of helix 69, known to be involved in subunit

association as well as in the transmission of structural rearrangements between the subunits during elongation (Yusupov, Yusupova et al. 2001), (Maivali and Remme 2004) (**Figure 35C**).

Though the binding sites for the two recycling factors seem to be the same, two differences were found. First ecRRF yielded stronger positive density (**Figure 35D,** positive density shown in blue**)** for domain I than ttRRF (**Figure 35D,** positive density shown in green) at a resolution of 8 Å. Second, binding of ttRRF to the *E .coli* ribosome caused a movement of helix 69 by 12 Å, revealed by both a positive and negative density peak in the map (see **Figure 35D**). Surprisingly, there are no difference peaks around helix 69 observed in the electron density map of ecRRF bound to the *E. coli* 70S, suggesting that ecRRF does not cause such a movement. However, the electron density map created with the 4.5Å dataset (data not shown) of the ribosome in complex with ttRRF do not show any difference peaks around helix 69 as in the case of ecRRF at 8Å. The positive density for domain I was slightly improved.

Discussion

1. Significance of the 3.5Å resolution structure of the *E. coli* 70S ribosome

As a result of this work the structure of the entire 70S ribosome at near atomic resolution has been obtained. The structure reveals a tremendous amount of information about the ribonucleo-complex in great detail, including the identification of magnesium ions that are known to be important for the structure and function of the ribosome (McCarthy 1962), (Zamir, Miskin et al.), (Spirin, Kisselev et al. 1963), (Gesteland 1966)). Due to the size of the ribosome (over 2,500,000 Dalton), the refinement of the 3.5 Å dataset is still progressing and will eventually show novel atomic details of the ribosome structure especially regarding the interaction between the two subunits.

2. Conformational changes

Superposition of the two ribosome molecules within the asymmetric unit (termed 70S#1 and 70S#2, as described above) revealed conformational changes occurring in the head and the spur of the 30S subunit as well as in the L1 arm, L7/L12 stalk and L11region of the 50S subunit.

As mentioned above, the movement observed between 70S #1 and #2 within the new crystal form resembles the “ratchet-linker” movement proposed by Frank and co-worker (Frank and Agrawal 2000). However, the conformational

change described here differs from the ratchet like movement, which was described as a merely rotation of the head region relative to the 50S subunit. The higher resolution of the x-ray structure presented here shows that in addition to the "sidewise" movement of the head region towards the E site, there is a 13 Å movement of the beak towards the 50S, describing a half circle (**Figure 31**). The closure of the A site observed here is reminiscent of that described by Frank's group in their reconstruction of 'locked' complexes (Valle, Zavialov et al. 2003). When the ribosome is in a 'locked' conformation, bearing a charged P site tRNA, binding of EF-G is inhibited. The factor could only bind to an 'unlocked' conformation of the ribosome, which bears a deacylated P site tRNA at that point. However, the fact that Frank and co-workers used A- and P site tRNAs and EF-G in their complexes may explain the discrepancies between the two observations. Alternatively, conformational changes described here and the ratchet-linker movement may reflect different states of the translation cycle.
The simultaneous movement of the spur in our crystal structure is very similar to that observed by the Frank group (Frank and Agrawal 2000), (Valle, Zavialov et al. 2003).

We also observed that the closing motion of the beak region of the 30S subunit is assisted by the L7/L12 stalk of the 50S subunit that moves towards the A site of the 30S subunit. This movement had not been observed by Frank and co-workers (Frank and Agrawal 2000), (Valle, Zavialov et al. 2003). Finally, the observation that the L1 arm would rotate towards the E site to assist the exiting tRNA has not been observed in our crystal structure. In fact, we observe the L1

arm moving away from the E site. One has to mention though that the region of the L7/L12 stalk as well as the L1 arm are disordered in our structure. This makes it possible that the observed movement of these regions may be an artifact.

Although the crystals presented in this work do not contain any ribosomal ligands as opposed to the experimental conditions used by Frank's group, we observe a similar movement. Since in the cryo-EM reconstruction the movement was observed in the presence of EF-G (Frank and Agrawal 2000), our results suggest that the conformational change occurring during translocation is an intrinsic property of the ribosome itself. This is in agreement with biochemical studies revealing that ribosomes have an innate translocase activity (Bergemann and Nierhaus 1983). With this in mind one could argue that the two different conformations described here could represent the pre- and post-translocational state. One could also speculate that the ribosomal subunits may be constantly moving with respect to each other until a factor locks them in a certain conformation. Alternatively, the possibility that the observed conformational changes are the result of crystal packing cannot be ruled out. However, the evidence presented above suggests that the movement of the subunits observed within these crystals is physiological relevant.

3. Kasugamycin

Kasugamycin (ksg) was reported to inhibit tRNA binding to the P-site during initiation (Okuyama, Machiyama et al. 1971), (Okuyama and Tanaka

1972), (Tai, Wallace et al. 1973). The inhibitory effect of ksg on initiation was found to be dependent on the mRNA template used (Kozak and Nathans 1972), (Okuyama and Tanaka 1972), (Hirashima, Childs et al. 1973), and to be lost in the presence of a leaderless mRNA (Chin, Shean et al. 1993). It has been recently hypothesized that the antibiotic may act by interfering with the binding of mRNA rather than acting on tRNA binding directly (Vila-Sanjurjo 2000). Evidence presented Vila-Sanjurjo and new results obtained by J. Michael Day (Day 2004) strongly suggest that the drug affects the binding of the mRNA in a region that overlaps the E and P site codons. Kasugamycin looses its ability to inhibit translation initiation in the presence of a leaderless mRNA (Chin, Shean et al. 1993), (Vila-Sanjurjo 2000). Interestingly, mutation of the E-site codon sequence has dramatic effects in the amount of inhibition by kasugamycin (**Figure 36A**). The fact that no clear consensus sequence emerged from this pattern of inhibition suggests that the inhibitory effect of kasugamycin might not depend on the primary sequence overlapping the E and P sites. Instead, inhibition may depend on the tertiary structures of the mRNA in this region as suggested by a previous structural analysis of the 30S subunit (Wimberly, Brodersen et al. 2000). This is shown in **Figure 36C, where** the 3' end of 16S rRNA is seen folding back into the mRNA channel, thereby acting as an mRNA analog (Wimberly, Brodersen et al. 2000). This is the only example of an mRNA observed in the ribosome at atomic resolution and shows that a hydrogen bond may exist between the cytosine at the second position of the E site codon and the phosphate group of the cytosine in at the third position (**Figure 36C**). This

interaction stabilizes the structural element of the mRNA backbone at the E site. Clearly, the potential to form a similar structural element should be affected by the identity of the sequence present at the E site. Moreover, there is also a clear effect of the first P site base on the extent of kasugamycin inhibition, as shown in **Figure 36B**. Superimposed in **Figure 36C** is the positive density of kasugamycin lying in between residues A794 and G926 (**Figure 34C & 34D**), and overlapping the P and E site as predicted (Vila-Sanjurjo 2000). In agreement with Vila-Sanjurjo's hypothesis (Vila-Sanjurjo 2000), the position of the drug also overlaps the path of the mRNA in this region, and is in close proximity to the structural element of the mRNA in the E site (**Figure 36C**). All these results taken together suggest that the identity of the mRNA sequence overlapping the P and E sites may have a profound effect on the structure of the mRNA in this region and, as a result, lead to dramatic consequences in the extent of kasugamycin inhibition. Prediction of the possible tertiary structures at the E site for all the mRNA constructs of **Figure 36A** is expected to reveal the details of how kasugamycin inhibits initiation during protein synthesis. In the future, one could even try to obtain crystal structures of ksg and different mRNA constructs complexed with the ribosome to understand the mechanism in molecular detail.

3. RRF

The RRF binding site observed in the structure is in agreement with a previous reported 12 Å cryo-EM structure of the *E. coli* RRF bound to the *E. coli*

ribosome (Agrawal, Sharma et al. 2004). These authors also reported a movement of helix 69 upon factor binding. A similar movement is observed in this work in the case of the 8A structure of ttRRF bound to the ribosome crystals. Surprisingly, this movement is not observed in the 4.5A structure. It is not clear why there is no movement of helix 69 observed in this study, since, in addition to the cryo-EM structure (Agrawal, Sharma et al. 2004), similar movement has been reported in a recent x-ray structure of the ecRRF domain I in complex with the *D. radiodurans* 50S subunit (Wilson, Schluenzen et al. 2005). A possible reason may be that the 4.5Å dataset is only 60% complete, so it still remains to be elucidated if movement of helix 69 actually occurs. We also need to explain why we do not observe any movement of helix 69 when ecRRF is bound to our crystals. The improvement of the difference maps might reveal the movement of helix 69 in our crystal structures.

A recent biochemical study of A. Kaji's laboratory (Raj, Kaji et al. 2005) revealed that there are two binding sites on the ribosome for RRF (Kiel, Raj et al. 2003). If this was true, then RRF might have to be translocated by EF-G to its second site, here it can trigger the conformational change of helix 69 that will eventually lead to subunit dissociation.

4. Final Thoughts

The achievement of obtaining crystals of the *E. coli* ribosome that diffract up to atomic resolution presented here has proven that it is possible to look at the

ribosome and the mechanism of protein synthesis in molecular detail. In addition, with the structures of the ribosome in complex with kasugamcyin and the RRF one was able to prove that the crystal form is suitable for performing soaking experiments. This observation increases our capabilities of obtaining atomic resolution structures of ribosomal complexes in different functional states. Combined, such structures in the future should allow for the recapitulation of the complete translational cycle at the ribosome in great molecular detail.

In summary, the results described in this thesis provide a significant advancement towards an understanding of the molecular mechanisms involved in protein synthesis.