Results

1. Structure determination and interpretation

In order to examine the structural basis for PY stabilization of 70S ribosomes against dissociation into 30S and 50S subunits, as a consequence of low magnesium concentration, a His-tagged version of Protein Y was soaked into *E. coli* 70S ribosome crystals. **Figure 7A** shows the crystals grown using the hanging drop technique resulting in different sizes dependent of the growth time. For these crystals, that belong to the spacegroup I422 the crystallographic parameters in **Table 1** were achieved. In the case of these crystals the resolution did not improve with the size of the crystals, the smaller crystals usually behaved much better. Diffraction data was collected to 11.4Å and 11Å for 70S empty ribosomes and 70S ribosomes in complex with PY, respectively. Although the crystals were grown under the exact same conditions as described in a previous paper by Vila-Sanjurjo et al. (Vila-Sanjurjo, Ridgeway et al. 2003) the unit cells were different in terms of their parameters. The new crystal form reveals a five - fold increase of the c-axes thereby holding five 70S ribosomes per asymmetric unit, as opposed to just one single 70S ribosome per asymmetric unit of the previously reposted crystals (**Figure 7B**). The position of the five different ribosomes, each containing one copy of PY within the asymmetric unit, can be seen in **Figure 8**. The new crystal form is related to the old form as can be seen by the fact that the large subunit data can be indexed as the small cell as shown in **Figure 9.**

The phase problem was solved by molecular replacement (**Figure 10**). The model used for solving the small unit cell (Vila-Sanjurjo, Ridgeway et al. 2003) derived from the atomic resolution structure of the *Deinococcus radiodurans* 50S subunit (Schluenzen, Zarivach et al. 2001) and the *Thermus thermophilus* 30S subunit (Wimberly, Brodersen et al. 2000). The model was altered to solve the structure of the new crystal form by molecular replacement (**Figure 10**). Briefly, an asymmetric unit containing five ribosomes was built by using five five asymmetric units of the smaller cell along the c axis. Since the two unit cells are cleary related rigid body refinement was used to verify that this search model could be used to phase the reflection data. Crystallographic refinement was carried out as described (Vila-Sanjurjo, Ridgeway et al. 2003).

The binding position of PY was observed in difference maps created with the datasets of the empty 70S and the 70S in complex with PY. PY binds to the 30S near the subunit interface and it is not in contact with the 50S. This finding supports previous biochemical data showing that PY binds only to the 30S subunit and the 70S ribosome but not to the 50S subunit (**Figure 11**) (Agafonov, Kolb et al. 1999). Helix 69 of the large 50S subunit, known to be involved in subunit association as well as in signal transmission between the two subunits (Yusupov, Yusupova et al. 2001) (Maivali and Remme 2004), is 10Å away from PY's electron density (asterix in **Figure 11A**). This suggests that Protein Y stabilizes the 70S ribosome without direct interaction with helix 69. Supporting the structural data chemical probing experiments using Dimethylsulfate (DMS) and kethoxal showed no altered reactivity of residues in helix 69. Most likely, the

binding of Protein Y to the small 30S subunit results in a higher affinity of the small subunit for the large 50S subunit.

Density resembling the shape of Protein Y (Ye, Serganov et al. 2002) (Rak, Kalinin et al. 2002) was found in all five ribosomes lying across the channel of the 30S subunit (**Figure 8**)**.** The N-terminal part of the NMR structure of Protein Y from A. Rak et al. (Rak, Kalinin et al. 2002) was used to dock the protein into the electron density. No density for the disordered C-terminal part was observed (**Figure 11C**)**.**

2. Comparison of PY, tRNA and mRNA binding sites

Superposition of tRNAs (Yusupov, Yusupova et al. 2001) onto the structure, reveals that PY binds mainly to the ribosomal P-site but also reaches into the A-site. This suggests that PY may actually inhibit tRNA binding to these two sites (**Figure 12A**). As can be seen in **Figure 12B**, the binding sites for Protein Y and mRNA (Yusupova, Yusupov et al. 2001) on the ribosome slightly overlap. However, binding experiments showed that Protein Y does not inhibit mRNA binding to the ribosome, suggesting that the two molecules have different binding sites at least in the absence of tRNAs.

No density for the protein within the ribosomal E-site was observed.

Because of the relatively low resolution (~11 Å) of this structure an

independent biochemical assay strengthening the results of the structure was required.

Using chemical probing, the occupancy of the two tRNA binding sites (A- and P site) by Protein Y was confirmed (performed by Anton Vila-Sanjurjo in the laboratory) again suggesting that the protein may indeed block tRNA binding to these two sites. The structure shows that PY's conserved positively charged backbone (Ye, Serganov et al. 2002) of the N-terminal region (**Figure 12C**) lies in close proximity to residues of the 16S rRNA that are universally conserved markers for tRNA binding to the A (A1493) and P site (G926, C1400) (Prince, Taylor et al. 1982) (Moazed and Noller 1989) and that are critical for ribosome function (Prince, Taylor et al. 1982) (von Ahsen and Noller 1995) (Vila-Sanjurjo and Dahlberg 2001). These interactions were probed by means of chemical probing.

The diagnostic site for tRNA binding to the A site, residue A1492 and A1493 of the 16S rRNA is protected in the presence of a tRNA (Moazed and Noller 1989) (Prince, Taylor et al. 1982) (**Figure 13A**, lane 3). Upon Protein Y binding to the ribosome, A 1492 is no longer protected and A1493 is enhanced which can be explained for the later by the fact that Protein Y binds near the backbone of residues 1495-1497 and not near the adenine base of 1493 (**Figure 13A**, lane 4). In the presence of tRNA and Protein Y the footprint for A1492 and A1493 is identical to the one observed with PY alone (**Figure 13A**, lane 5) suggesting that the protein inhibits tRNA binding to the A site.

By looking at the structure, PY residues Lys86, Lys79 and Arg82 were

found in close proximity of the universally conserved P site residues G926 and C1400 of the 16S rRNA in the P-site and of C1402, within a universally conserved wobble pair (Vila-Sanjurjo and Dahlberg 2001), respectively (**Figure 12C**). In agreement with this chemical probing results showed that G926 and C1400 are protected upon binding of PY (**Figure 13B**, lane 3 and **Figure 13C**, lane 3, respectively) suggesting that the Protein binds in a very similar way to the P site as a tRNA (**Figure 13B**, lane 4 and **Figure 13C**, lane 4, respectively) thereby blocking tRNA binding to this site (**Figure 13B**, lane 5 and **Figure 13C**, lane 5, respectively).

In agreement with the structural data Protein Y chemical probing results show no effect on tRNA binding to the ribosomal E-site as revealed by the fact that the protection of C2394 of the 23S rRNA upon tRNA binding to the E-site (Moazed and Noller 1989) is not altered by the addition of PY (**Figure 13D**, lane 3 and lane 5)**.**

3. Inhibition of P site binding

The structural data indicate that PY's binding site primarily overlaps with the P site and to a lesser extent with the A site. This suggests that PY may function as an inhibitor of tRNA binding to the P site and, as a consequence inhibits tRNA binding to the A site. This issue was investigated further by means of filter binding assays using different mRNA constructs that encode for $tRNA^{fMet}$ at the ribosomal P site. In the presence of a leaderless ^{33}P labeled mRNA, which

is only able to bind to the ribosome if a P site tRNA is present, PY can inhibit the binding of a deacylated $tRNA^{fMet}$ to the ribosomal P site (**Figure 14A**). The same result was obtained while using a canonical Shine-Dalgarno (SD) sequence and ^{33}P labeled $tRNA^{fMet}$. The inhibitory effect of PY in the later experiment was enhanced by changing the temperature from 37°C to 16°C consistent with PY's function during cold shock (**Figure 14B**).

4. PY's effect on translation initiation

During translation there are only two stages when the ribosome has only one tRNA bound to its P site. The first one is translational initiation during which the initiator tRNA, fMet $tRNA^{fMet}$ is brought to the P site with the help of Initiation Factor (IF) 2 / GTP (Brock, Szkaradkiewicz et al. 1998). The second stage is translational termination during which the peptide is released from the P site leaving behind a deacylated tRNA at the P site (Janosi, Hara et al. 1996).

Since it has been proposed that translational arrest during cold shock primarily occurs through inhibition of translational initiation (Gualerzi, Giuliodori et al. 2003) the possibility that PY may function at the stage of initiation seemed a likely one.

This hypothesis was investigated by means of filter binding assays using ^{35}S labeled fMet $tRNA^{fMet}$. PY's ability in preventing tRNA binding to the P site was tested in the presence and absence of initiation factors (IF1, IF2, IF3) at 37°C as well as 16°C, mimicking cold shock conditions. In addition the presence

of polyamines in the buffer, mimicking *in vivo* like conditions (**Table 2**, (Nierhaus, Spahn et al. 2000)) was very crucial in PY's ability to function.

In the presence of polyamines, PY is able to inhibit fMet $tRNA^{fMet}$ binding to the ribosomal P site in the absence of initiation factors (**FIGURE 15A, B**: white columns). In the presence of the initiation factors (**Figure 15A, B**: black columns) PY does not inhibit fMet $tRNA^{fMet}$ binding to the P site at 37°C, whereas, consistent with its role during cold shock, PY significantly impairs tRNA binding at 16°C. These results suggest that PY is unable to block initiation at normal temperatures but gains this ability at low temperatures.

Strikingly, in the absence of polyamines PY looses its ability to inhibit initiation at 16°C when IFs are present as can be seen in **Figure 15D**. Initiation is not inhibited by PY at 37°C in the absence of polyamines, but is able to inhibit fMet $tRNA^{fMet}$ binding to the P-site in the absence of initiation factors (**Figure 15C**).

5. The effect of PY on IF-dependent ribosome dissocation

Translation initiation requires the dissociation of the 70S ribosome into its two subunits. In bacteria IF3 prevents the 30S subunit from associating with the 50S until IF2/GTP brings along fMet $tRNA^{fMet}$ (Agafonov, Kolb et al. 1999) (Brock, Szkaradkiewicz et al. 1998). After binding of the initiator tRNA, IF2/GDP dissociates from the ribosome. There is no unique function attributed to IF1, a factor that binds to the A site, other than that it promotes IF2 function (reviewed

in (Karimi, Pavlov et al. 1998) (Gualerzi and Pon 1990) (Moreno, Drskjotersen et al. 1999)).

Since PY and IF3 have opposite effects of ribosome association, it seemed reasonable that PY might be displaced by IF 3 once normal growth conditions start to reoccur. In order to investigate that IF3 could override the stabilization of 70S ribosomes by PY different ribosome complexes were run on sucrose gradients resulting in ribosome profiles. **Figure 16A** and **16B** only differ again in the buffer-systems that were used. In **16A** polyamines were present whereas in **Figure 16B** they were absent. 70S ribosomes in the absence (**Figure 16B & 16B**: gradient 1) and presence of PY (**Figure 16A &16B**: gradient 4) result in a single peak on the profile. After the addition of IF3 the 70S dissociates into the 30S and 50S subunit as can be seen in the profile 2 in **Figure 16A & 16B**. The same is true when IF1 is added in addition to IF3 (**Figure 16A & 16B**: gradient 3). Ribosomes pre-incubated with PY are completely resistant to dissociating function of IF3 (**Figure 16A**: gradient 5) in the presence of polyamines and are only slightly dissociated in the absence of polyamines (**Figure 16B**: gradient 5). The addition of IF1 does not change this result in the presence of polyamines (**Figure 16A**: gradient 6). In the absence of polyamines Protein Y looses its ability to compete with IF 3 when IF1 is around (**Figure 16B**: gradient 6). Notably, in the absence of polyamines IF3 together with IF1 works in tandem to override PY's function resulting in a new role for IF1 in the dissociation process of the two subunits.

Comparison of the binding position of Protein Y and IF1 (Carter, Clemons

et al. 2001) reveal steric overlaps between the two proteins (**Figure 16C**) suggesting that the proteins are competing for binding to the 30S subunit. Since the structural information available for IF3 (McCutcheon, Agrawal et al. 1999) (Dallas and Noller 2001) is not suitable for performing such superposition it remains unclear if IF3 and PY share same binding sites at the 30S subunit, though it is tempting to speculate it after the results shown here.

6. Inhibition of A site binding

During the elongation cycle the ribosomal P site is always occupied by a peptidyl-tRNA. Strikingly, based on experiments lacking a peptidyl-tRNA at the P site Spirin and colleagues concluded that Protein Y inhibits *in vitro* translation at the elongation step by interfering with tRNA binding to the ribososomal A site (Agafonov, Kolb et al. 2001). Although the previous initiation experiments clearly showed that PY is able to inhibit initiation the structural and biochemical data presented so far are also in agreement with the fact that Protein Y may inhibit tRNA binding to the A-site. To further investigate the proposal by Spirin and co-workers (Agafonov, Kolb et al. 2001) A site binding experiments in the presence of a peptidyl-tRNA at the P-site were performed, thereby mimicking the state of the ribosome prior to A site decoding. The experiment was performed at 37°C as well as 16°C, mimicking cold shock conditions. In the presence of ^{35}S labeled fMet $tRNA^{fMet}$ (used as a peptidyl tRNA) at the P site, Protein Y is unable to inhibit A site binding of ^{3}H labeled Phe $tRNA^{Phe}$/ EF-Tu/ GTP at both

temperatures (**Figure 17A, B**: columns 1 and 2)**.** Protein Y's ability to block P site tRNA binding was used as a control for the protein's functionality: Protein Y is able to inhibit ^{35}S labeled fMet $tRNA^{fMet}$ binding to the P site in the presence and absence of IFs at 16°C. It is only able to inhibit P site binding in the absence of IFs at 37°C (**Figure 17A, B** columns 3-6)

This suggests that PY does not interfere with *in vitro* protein biosynthesis during the elongating step, a result that is in line with reports excluding a block in elongation as a cause of the translational arrest observed during cold shock (Maki, Yoshida et al. 2000)**.**

Discussion

Bacteria rarely encounter environmental conditions that allow them to grow exponentially. In fact, bacteria are frequently exposed to long periods of starvation due to stress conditions like temperatures changes, pressure, osmotic stress or changes in oxygen availability.

This work reveals the first mechanism on how translation is controlled during cold shock in *E. coli*. Shortly after the cells sense a drop in temperature translation of all but a subset of ~26 proteins (VanBogelen and Neidhardt 1990), (Gualerzi, Giuliodori et al. 2003), (Giuliodori, Brandi et al. 2004), including Protein Y is inhibited at the stage of initiation. By blocking the translation of all proteins but the essential cold shock proteins, the survival of the cell in the cold is ensured (Gualerzi, Giuliodori et al. 2003).

In this work it was shown that during cold shock Protein Y binds to the ribosomal P site, thereby inhibiting translational initiation. PY was found to block the interaction between ribosomes and tRNAs and possibly mRNAs (**Figure 14**). In addition, consistent with PY's role during cold shock, the protein is able to compete with one or more conserved initiation factors (**Figure 16**) at 16°C thereby inhibiting translational initiation in the cold. For initiation to occur at 37°C PY would have to be displaced by one if the initiation factors since the initiator tRNA (fMet $tRNA^{fMet}$) by itself does not have this ability (**Figure 15A**, -IF/+PY).

In order to ensure the expression of cold shock proteins PY's inhibitory function might be overridden by cis-acting elements in cold-shock mRNAs or cold

shock proteins such as CspA as well as the increased synthesis of initiation factors in the cold (Giuliodori, Brandi et al. 2004). By blocking translation of all proteins but the one of cold shock proteins all translation factors are involved in the expression of cold-shock proteins thereby ensuring survival of the cell in the cold (Gualerzi, Giuliodori et al. 2003). Shortly after cells have adapted to the cold more ribosomes are entering the translational pool (Thieringer, Jones et al. 1998) resuming translation at a much slower rate as during normal growth conditions (Gualerzi, Giuliodori et al. 2003).

However, during cold shock a substantial number of 70S monosomes (Uchida, Abe et al. 1970) are stabilized by PY, following its postulated role of being a storage protein (Agafonov, Kolb et al. 1999), (Agafonov, Kolb et al. 2001). It is thereby able to protect several conserved element within the ribosome from degradation: subunit interface, key residues of the A and P site, and possibly the 3' end of the 16S rRNA.

Once optimal growth conditions are being restored Protein Y is no longer able to compete with IFs. PY dissociates from the ribosome after which is will be degraded, since the protein is not found at normal growth conditions (Agafonov, Kolb et al. 1999) (see also **Figure 18** for a model of PY).

The use of concentrations of ions and polyamines occurring *in vivo* (see Table 2 (Nierhaus, Spahn et al. 2000)) were found to be very important for the rate and accuracy of protein synthesis. Different buffer systems (conventional buffers versus polyamine buffers) resulted in different binding positions for tRNAs as shown by cryo-EM (Agrawal, Penczek et al. 1999). The right ionic conditions

were also important in this study, where the use of different buffer systems (+/- polyamines) revealed different results for PY's function. Only in the presence of polyamines PY was able to inhibit translation initiation. This turned out to be another example for the importance of correct *in vivo* near ionic conditions that if used will prevent artifacts.

An increase in temperature primarily leads to improper protein folding or denaturation of proteins. A specific set of heat shock proteins is induced under these conditions that work as molecular chaperones, assisting protein folding at higher temperature. Heat shock proteins, in *E. coli* require a specific sigma factor for their transcription. On the contrary, there is no evidence that proteins that are induced upon cold shock require any kind of σ–factor. Instead cold shock proteins seem to use already existing resources in the cell at the time of temperature downshift, as in the case of CspA, a RNA a chaperone and the major cold shock protein in *E. coli* (Giuliodori, Brandi et al. 2004). CspA is regulated at a number of levels, like transcription, mRNA stability and translation (Giuliodori, Brandi et al. 2004). Cis-acting elements might also be important for the control the expression of Protein Y's and other cold shock proteins in the cold. The increased synthesis of IFs observed during cold shock (Gualerzi, Giuliodori et al. 2003) might also help to be important for the expression of PY in the cold.

In addition to Protein Y, there are several other proteins that were found in bacteria to interact with the ribosome during environmental stress. RMF (ribosome modulation factor) induces the formation of the 100S ribosomes

(Yoshida, Yamamoto et al. 2004), (Yoshida, Maki et al. 2002), which are formed the dimerization of two 70S ribosomes and is yet is another storage form of the ribosome during stress. YhbH binds exclusively to 100S ribosomes (Maki, Yoshida et al. 2000) and has 40% sequence similarity to PY suggesting that the binding site of these two proteins on the ribosomes might be the same.

In addition there are about 50 sequence homologues of PY found in known bacteria genomes and similar proteins have been found in plant genomes (*Arabidopsis thaliana* and *Spinacia oleracea*) (Ye, Serganov et al. 2002). The PY homolog in spinach chloroplasts has been shown to associate with the small subunit of the plastid ribosome as well as with that of *E. coli* monosomes, suggesting that similar to PY it also interacts with conserved regions of the ribosome. In addition, another homolog was found in cyanobacteria that is only expressed in the dark. In cyanobacteria this is the moment when most of the translation is inhibited, mimicking a state of stress, suggesting a similar role as PY for this protein (Tan, Varughese et al. 1994).