Chapter 2

Functional proof for the translocating activity of Hsp15 in the recycling of aborted ribosomal 50S subunit-nascent chain tRNA complexes†

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† This chapter is based on “Recycling of aborted ribosomal 50S subunit-nascent chain tRNA complexes by the heat shock protein Hsp15.” Linhua Jiang, Christiane Schaffitzel, Rouven Bingel-Erlenmeyer, Nenad Ban, Philipp Korber, Roman I. Koning, Daniël C. de Geus, Jasper R. Plaisier, Jan Pieter Abrahams J. Mol. Biol. (2008). It focuses on Daniël de Geus’ contribution to this paper, which was to provide functional proof for a mechanism that was deduced from structural data.
Abstract

When heat shock prematurely dissociates a translating bacterial ribosome, its 50S subunit is prevented from re-initiating protein synthesis by tRNA covalently linked the to unfinished protein chain that remains threaded through the exit tunnel. Hsp15, a highly up-regulated bacterial heat shock protein, is essential for re-activating such dead-end complexes. Here we show with functional assays that Hsp15 translocates the tRNA moiety from the A- to the P-site of stalled 50S subunits to expose the aminoacylester bond between tRNA and nascent chain to ribosome mediated cleavage. These assays complement cryo-EM reconstructions of the complex of the 50S•nc-tRNA sub-unit in the absence and in the presence of Hsp15. By stabilising the tRNA in the P-site, Hsp15 indirectly frees up the A-site, allowing a release factor to land there and cleave off the tRNA. Such a release factor must be stop codon independent, suggesting a possible role for a poorly characterised class of putative release factors that are up-regulated by cellular stress, lack a codon recognition domain and that are conserved in eukaryotes.
2.1 Introduction

Heat shock up-regulates many proteins that function as chaperones or as proteases. Heat shock also increases the transcription of the small heat shock protein Hsp15, which is an RNA/DNA binding protein. It targets aborted ribosomal 50S subunits rather than misfolded proteins\(^1\). Its ~50-fold transcriptional increase is even higher than the upshift in expression of well-characterized heat shock proteins such as GroEL/ES, DnaK and ClpA, indicating the high relevance of Hsp15 for adapting to thermal stress\(^2\).

Translating ribosomes can dissociate prematurely upon heat shock, resulting in 50S subunits that carry tRNA covalently attached to the nascent chain (nc-tRNA) of an incomplete protein that is threaded through the 50S exit tunnel\(^3\). These 50S•nc-tRNA subunits cannot re-initiate protein synthesis and their accumulation would constitute a problem for the cell. In the intact 70S ribosome, the tRNA is released from the nascent chain by a release factor, which binds in the vacant A-site to cleave the peptidyl ester bond. All well-characterised release factors are stop codon dependent\(^3-5\), yet the release factor that recycles blocked 50S subunits must be stop codon-independent, as there is no stop codon in the dissociated 50S subunit.

Rescuing such blocked 50S subunits requires the presence of Hsp15, which specifically binds blocked 50S•nc-tRNA ribosomal subunits with high affinity (\(K_d < 5\) nM), while its affinity for empty, functional 50S subunits is

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\(^1\) Presumably tRNA attached to a short peptide would still dissociate, hence we use the term ‘nc-tRNA’ throughout the chapter to distinguish it from tRNA attached to very short peptides.
significantly lower\textsuperscript{6}. It was established that Hsp15 is not a release factor, since the 50S•nc-tRNA•Hsp15 is stable and the tRNA moiety is not released\textsuperscript{7}. It remained unclear how Hsp15 discriminates between active and aberrantly terminated 50S subunits and how it contributes to recycling blocked, non-functional 50S•nc-tRNA complexes.

In this chapter, puromycin nascent chain release assays are described which were used to prove a conjecture based on electron microscopy (EM) 3D reconstructions of the complexes involved. These EM results are reported in detail elsewhere\textsuperscript{8}. Briefly, the structure of the complex of the 50S•nc-tRNA subunit both in the absence and presence of Hsp15 were determined by cryo-EM and single particle analysis to resolutions of 14 and 10 Å, respectively. The 3D models of these complexes were reconstructed and X-ray structures of 50S, tRNA and Hsp15 were subsequently fitted into EM density maps as described elsewhere\textsuperscript{8}. To summarize the cryo-EM results, the 50S•nc-tRNA reconstruction revealed clear additional density located at the A site (Figure 1A), corresponding to the tRNA covalently attached to the nascent polypeptide chain that is extending through the ribosomal exit tunnel. In contrast, the tRNA appears at the P site in the 50S•nc-tRNA•Hsp15 reconstruction (Figure 1B).
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Figure 1  Reconstructions of: a) the 50S•nc-tRNA complex (the density of tRNA is in cyan, 14 Å resolution) and b) the 50S•nc-tRNA•Hsp15 complex (the density of tRNA is in cyan and of Hsp15 in blue, 10 Å resolution). The Central Protuberance (CP), the L1 and L7/L12 domains and the P- and A-sites are indicated.

2.2 Experimental procedures

2.2.1 Preparation of 50S•nc-tRNA complexes

The plasmid pUC19Strep3FtsQSecM was transcribed in vitro and translated in a membrane-free E. coli cell extract as previously described. The translation mix was loaded directly onto a 38 ml sucrose gradient (10 – 50% sucrose in 50 mM Hepes-KOH, 100 mM KOAc, 0.3 mM Mg(OAc)$_2$, pH 7.5) and centrifuged for 15 hours at 23000 rpm, 4°C in a SW32 Ti rotor (Beckman Coulter). The 50S fraction was loaded onto a 300 µl Strep-Tactin sepharose column (IBA, Göttingen Germany) equilibrated with buffer 1 (20
mM Hepes-KOH, 150 mM NH₄Cl, 1 mM Mg(OAc)₂, 4 mM β-mercaptoethanol, pH 7.5) at 4°C, eluted with 2.5 mM desthiobiotin in buffer 2 (20 mM Hepes-KOH, 150 mM NH₄Cl, 12 mM Mg(OAc)₂, 4 mM β-mercaptoethanol, pH 7.5) and pelleted by ultracentrifugation (3 h, 55000 rpm, 4°C, TLA-55 rotor (Beckman)). The 50S•nc-tRNA (50S-nc) pellet was dissolved in buffer 2 by gentle shaking on ice.

2.2.2 Purification of Hsp15

The plasmid pTHZ25¹ was transformed in *E. coli* BL21(DE3) and the cells were cultured as described. Cells were ruptured by two passages through an EmulsiFlex-C5 homogenizer (Avestin) at 15000 psi and the lysate cleared by ultracentrifugation (1h, 18000 rpm, 4°C, Ti70 rotor (Beckman)). The supernatant was loaded onto a Q sepharose FF column (GE Healthcare) and a phenyl-sepharose column (GE Healthcare) as described¹. The purified protein was dialyzed against 30 mM Hepes-KOH, 1 mM EDTA pH 7.0, concentrated with a Centriplus concentrator (MWCO 3 kDa, Amicon), flash-frozen and stored at -80 ºC.

2.2.3 Binding assays of Hsp15

15 μg 50S-nc, 50S and 70S were incubated in a 1:1 and 1:10 molar ratio with purified Hsp15 in buffer 3 (20 mM Hepes-KOH, 100 mM NH₄Cl, 25 mM Mg(OAc)₂, pH 7.5) on ice for 30 min. The mix was centrifuged 5 min at 14000 rpm in a table top centrifuge at 4°C and then loaded onto a 1.5 ml
sucrose cushion (30% w/v sucrose in buffer 3). The ribosomes and ribosomal subunits were pelleted by ultracentrifugation (5 h, 55000 rpm, 4°C, TLA-55 rotor (Beckman)). The ribosomal pellet was quantified and loaded onto a 16% SDS gel.

2.2.4 Puromycin assay

60 nM 50S•nc-tRNA•Hsp15 complex in buffer 2 (or buffer 2 with increased Mg\(^{2+}\) concentration to 100 mM to favour Hsp15 dissociation) was incubated with 2 mM puromycin for 3 h at 37 °C. Samples at 45 min intervals were withdrawn, mixed with an equal volume of loading buffer and separated on a low-pH SDS-based Tris-acetate gel to minimize hydrolysis of the ester bond linking tRNA to the nascent chain\(^9\). Immunodetection of the nascent chain was carried out on PVDF membrane using a Strep-tag monoclonal antibody conjugated to horseradish peroxidase (IBA, Göttingen Germany). Detection was performed by electrochemiluminescence and spots on the X-ray films were quantified densitometrically.

2.3 Results and Discussion

2.3.1 Generation of stable 50S•nc-tRNA complexes

Stable, homogenous 70S•nc-tRNA complexes were generated by \textit{in vitro} transcription and \textit{in vitro} translation using the plasmid pUC19Strep3FtsQSecM with an N-terminal triple Strep-tag for affinity purification\(^7\). To span the ribosomal exit tunnel, the FtsQ sequence and the
17 amino acids-long SecM translational arrest motif were C-terminally fused to the affinity tag. The SecM peptide interacts tightly with the ribosomal tunnel\(^\text{10}\) and thereby significantly stabilizes the 70S•nc-tRNA complexes, without the need of using chloramphenicol antibiotic. After \textit{in vitro} translation, the translating ribosomes were loaded onto a sucrose gradient with low concentration of magnesium ions causing dissociation of the 70S•nc-tRNA complexes into 50S•nc-tRNA and 30S (Figure 2A) complexes. The 50S•nc-tRNA complexes were further purified and separated from empty 50S using a Strep-Tactin sepharose column and finally pelleted by ultracentrifugation. The complex with Hsp15 was reconstituted by adding a 20-fold molar excess of Hsp15. Binding assays confirmed that Hsp15 neither binds 70S ribosomes nor empty 50S subunits under the assay conditions (Figure 2B). However, a low level of binding of Hsp15 to empty 50S subunits was observed, when the sucrose cushion was omitted from the sedimentation assay (not shown). This is in agreement with the previously described non-specific nucleic acid binding activity of Hsp15\(^\text{1}\). Hsp15 only had a high affinity for 50S subunits when they contained nc-tRNA. Also, Hsp15 had to be present in a 1:10 molar ratio (Figure 2B).
Figure 2

a) Preparation of 50S•nascent chain-tRNA complexes (50S-nc/50S•nc-tRNA).
Sucrose gradient profile of an in vitro translation reaction in the presence of 0.3 mM Mg(OAc)2. The two peaks (50S and 30S) are analyzed on a Coomassie-stained SDS gel. The presence of the nascent chain in the 50S is shown by Western blotting (left side) using Streptactin-alkaline phosphatase conjugate. The upper band at ~34 kDa corresponds to nc-tRNA, the lower band to the nascent peptide alone.

b) Binding assay of Hsp15. Binding of purified Hsp15 to 50S-nc, to 50S and to 70S was analyzed by ribosomal pelleting through a sucrose cushion. As a control (indicated with c), 50S and 70S was loaded alone. Hsp15 did not migrate through the sucrose cushion (not shown). Hsp15 was added in a 1:1 and 1:10 molar ratio. Hsp15 was found only in the pellet of 50S-nc as indicated with arrows in lanes 1 and 2; in the 1:1 molar ratio somewhat less Hsp15 was recovered. As a positive control, 100 ng Hsp15 was loaded onto the SDS gel.
2.3.2 Functional assay of Hsp15 induced tRNA translocation

The P-site specific antibiotic puromycin is a functional equivalent of a stop codon independent release factor. Mimicking the 3’ end of aminoacyl tRNA at the A-site, it binds at the A-site and allowing the ribosome to cleave off P-site tRNA from the nascent chain, which is transferred to the A-site moiety. Puromycin is used in functional assays to distinguish P-site tRNA from A-site tRNA and establish A-site occupancy. It was established that puromycin abolishes binding of Hsp15 to 50S•nc-tRNA complexes in cell extracts6. Presumably, puromycin released the tRNA from the 50S subunit and the resulting empty 50S subunits would no longer have a high affinity for Hsp15. This observation already indicated that the tRNA must reside in the P-site in the 50S•nc-tRNA•Hsp15 complex in cell extracts. Here we show that no additional factors were involved: also in highly purified 50S•nc-tRNA•Hsp15 samples, puromycin was able to cleave off the nascent chain (Figure 3).

N-acetylated Phe-tRNA_{Phe} is a nc-tRNA homologue that can freely diffuse into and out of the P-site of 50S subunits, where can react with A-site bound puromycin. This reaction proceeds optimally at 100 mM Mg^{2+} in isolated 50S subunits, but is slower at lower Mg^{2+} concentrations12. However, for the 50S•nc-tRNA•Hsp15 we found the opposite effect: raising the Mg^{2+} concentration reduced the puromycin reactivity.
Figure 3  a) Puromycin reaction of 50S•nc-tRNA•Hsp15 at 37 °C in 12 mM and 100 mM Mg\(^{2+}\). Controls without puromycin do not show any cleavage of the ester bond between tRNA and nascent chain, even after 3 hours of incubation. At the outset of the reaction, there is already a substantial amount of released nascent chain present (lower band). b) The negative natural logarithm of the remaining nc-tRNA was plotted against the incubation time. In a first order reaction this is expected to be a straight line, which we indeed observe for 2-3 hours after initiating the reaction. The data shown in a) are plotted. The puromycin induced cleavage of nc-tRNA was determined by measuring the increase of the intensity of the band corresponding to the released nascent chain as a fraction of the total intensity corresponding to nascent chain (whether bound to tRNA or not). The higher reactivity at 12 mM Mg\(^{2+}\) can be explained by the fixation of tRNA at the P-site by Hsp15 at this Mg concentration. The experiments were performed in duplicate.
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The cryo-EM structure provided a straightforward explanation of this observation. At 100 mM Mg\(^{2+}\), Hsp15 dissociates more easily from 50S•nc-tRNA complexes. If Hsp15 is essential for stabilising the tRNA moiety in the P-site, as suggested by the structures, its dissociation from the 50S•nc-tRNA•Hsp15 complex should result in a relocation of the tRNA to the A-site, where it cannot be cleaved by puromycin.

2.3.3 Recycling ribosomal complexes by Hsp15: discussing the model

Translational reactivation of a heat shock aborted 50S•nc-tRNA complex requires removal of the nc-tRNA by severing of the aminoacylester bond between these moieties. In the cell, this hydrolysis requires the tRNA to be located in the P-site and a release factor to bind at the vacant A-site. In the absence of Hsp15, the tRNA moiety of nc-tRNA, although being somewhat disordered, was clearly located in the A-site (Figure 1A). This A-site location of the tRNA moiety is further corroborated by a puromycin assay (Figure 3). At first sight this is a surprising result, as in the complete 70S ribosome, peptidyl tRNA has a preference for the P-site. However, in the 70S complex, peptidyl tRNA is stabilized at the P-site by extensive contacts with the mRNA, 16S RNA and protein residues of the 30S subunit (e.g., Ref. 13) and these interactions are obviously absent in the 50S•nc-tRNA complex. On the other hand, the A-site location of the tRNA is stabilized by additional interactions with residues of the 50S subunit that lie outside the peptidyl transfer center, e.g. helix 38 of the ‘A-site Finger’. Apparently, in the absence of the 30S subunit, these interactions are strong enough to direct
the tRNA moiety in the 50S•nc-tRNA complex to the A-site. Our observation explains why the 50S•nc-tRNA complex cannot be recycled: a tRNA moiety in the A-site prevents release factors from binding and severing the aminoacylester bond between the tRNA and the nascent chain. Hsp15 located the tRNA in the P-site. Locked in the P-site, the CCA end of the nc-tRNA is optimally positioned in the peptidyl transferase centre for hydrolytic attack of its aminoacylester bond by a release factor. The translocation of the tRNA to the P-site in the Hsp15 containing complex is in full agreement with the puromycin assay (Figure 3) and with puromycin sensitivity of dissociated translating ribosomes in cell lysates. Translocation of the nc-tRNA from the A- to the P-site would allow a release factor to bind in the A-site. In the 70S ribosome, this translocation requires energy: EF-G hydrolyses GTP in the process. Our results indicate that in the absence of interactions with the 30S subunit, the binding energy of the Hsp15 to the 50S•nc-tRNA complex is sufficient to induce translocation.

Which release factor cleaves the aminoacylester bond between tRNA and nascent chain in the 50S•nc-tRNA•Hsp15 complex? All well-characterized release factors interact with translating ribosomes and mimic a tRNA molecule. They all have a stop-codon recognizing domain at one end and a GGQ peptidyl hydrolase domain at the other end, which interacts with the peptidyl transferase center of the ribosome. In the blocked 50S•nc-tRNA•Hsp15 complex there is no stop-codon. The putative 15 kD, 140 aminoacid protein with unassigned function encoded by the yaeJ gene in E. coli is a likely candidate to serve as a release factor for 50S*nc-tRNA-
Hsp15 complexes. In *E. coli yaeJ* is transcribed immediately ahead of *cutF/nplE*, a factor involved in the extracytoplasmic stress response; both apparently belong to the same stress-induced operon\textsuperscript{16}. YaeJ contains the conserved GGQ peptidyl hydrolase domain, but lacks a stop-codon recognizing domain. Due to the presence of the GGQ motif, YaeJ is placed in the same cluster of orthologous groups as the release factors RF1 and RF2\textsuperscript{15}. Thus, YaeJ could bind to the A site of the 50S\textsuperscript{nc}-tRNA\textsuperscript{Hsp15} complex and hydrolyze the peptidyl-tRNA ester bond without needing a stop codon recognizing domain.

Residues 10 to 112 of YaeJ have a significant 29\% identity and 55\% similarity with the small human protein ICT1, indicating both share the same fold. ICT1 is a 23.6 kD protein with unknown function, but it becomes more highly expressed upon neoplastic transformation of colon epithelial cells\textsuperscript{17} and is predicted with high significance (P>0.9) to be targeted to mitochondria\textsuperscript{18}. On the basis of its GGQ peptidyl hydrolase domain, ICT1 is classified as a putative release factor, even though, like yaeJ, it lacks an anticodon recognizing domain. If ICT1 recycles stress-induced mitochondrial 50S\textsuperscript{nc}-tRNA complexes, this might explain its upregulation in neoplastic transformation, which is a process requiring the inhibition of apoptosis, for instance by the reduction of mitochondrial stress. Details will differ, as we did not find a eukaryotic homologue of Hsp15.

In this work, the SecM peptide was chosen for practical reasons – to stabilize the complexes by interaction of the nascent polypeptide with the ribosomal tunnel. This is a very unique peptide that causes translational arrest, prompting the question whether it is valid to extrapolate the results to
the general case. However, the presence of the SecM peptide is necessary, though not sufficient, for ribosome stalling: puromycin can still efficiently attack a tRNA carrying the SecM peptide\(^7\), indicating P-site location of the tRNA moiety in the 70S complex and a functional peptidyl transfer centre. Full stalling by the SecM peptide additionally requires the presence of Pro-tRNA\(^{\text{Pro}}\) at the A site\(^{19}\). In the stalled 50S subunit, this second condition cannot be met for obvious reasons, so the SecM-stalled nc-tRNA\(^{\text{•}50\text{S}}\) complex most likely does represent the general case of a heat-shocked 50S-peptidyl tRNA complex. In addition, the finding that Hsp15 only has specificity for 50S ribosomal subunits with a tRNA, regardless of the sequence of the nascent chain\(^6\), further supports the general relevance of our finding. In conclusion, we propose that Hsp15 rescues heat-induced abortive 50S\(^{\text{•}\text{nc-tRNA}}\) subunits by fixing the tRNA moiety to the P-site, regardless of the nature of the nascent chain (Figure 4). This allows a (specialized) release factor to bind at the A-site and cleave the aminoacylester bond between tRNA and nascent chain. The cleavage allows tRNA and nascent chain to diffuse away and the 50S particle to become translationally active again.
Figure 4  Rescue cycle of the stalled ribosomal 50S subunit. Heat shock can erroneously dissociate a translating ribosome into a 30S subunit and a blocked 50S subunit carrying a tRNA linked to the unfinished nascent chain (upper right). Here we show that in these stalled 50S•nc-tRNA complexes, the tRNA is located at the A-site (bottom right) and that the small heat shock protein Hsp15 translocates the tRNA to the P-site (bottom left), where it can be liberated by a release factor (top left).
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