The 2'-OH group of the peptidyl-tRNA stabilizes an active conformation of the ribosomal PTC

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The ribosome accelerates the rate of peptidyl transfer by $>10^6$ -fold relative to the background rate. A widely accepted model for this rate enhancement invokes entropic effects whereby the ribosome and the 2'-OH of the peptidyl-tRNA substrate precisely position the reactive moieties through an extensive network of hydrogen bonds that allows proton movement through them. Some studies, however, have called this model into question because they find the 2'-OH of the peptidyl-tRNA to be dispensable for catalysis. Here, we use an *in vitro* reconstituted translation system to resolve these discrepancies. We find that catalysis is at least 100-fold slower with the dA76-substituted peptidyl-tRNA substrate and that the peptidyl transferase centre undergoes a slow inactivation when the peptidyl-tRNA lacks the 2'-OH group. Additionally, the 2'-OH group was found to be critical for EFTu binding and peptide release. These findings reconcile the conflict in the literature, and support a model where interactions between active site residues and the 2'-OH of A76 of the peptidyl-tRNA are pivotal in orienting substrates in this active site for optimal catalysis.

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Introduction

The ribosome is the macromolecular machine responsible for protein synthesis in all domains of life. During protein synthesis, peptide-bond formation takes place on the large subunit of the ribosome when the nucleophilic α -amino group of the aminoacyl-tRNA attacks the carbonyl carbon of the ester bond that attaches the nascent peptide to the peptidyl-tRNA. Although the reactivity of amines with esters is intrinsically high, the ribosome nonetheless accelerates the rate of peptide-bond formation by $>10^6$ -fold (Sievers *et al*, 2004). The ribosome, like all enzymes, is theoretically

capable of employing a number of different strategies to stabilize the transition state and promote catalysis. For instance, it may add or abstract protons during the reaction, orient substrates in an optimal position for the reaction to proceed, and use remote binding interactions to introduce unfavourable contacts in the active site that are relieved by the formation of the transition state (Doudna and Cech, 2002).

What contribution do ribosomal moieties make to catalysis of this important reaction? The high-resolution crystal structures of the large subunit, in complex with substrates, products and transition-state analogues, indicate that the PT centre is composed primarily of conserved nucleotides found in the central loop of domain V of the 23S RNA (Ban *et al*, 2000; Nissen *et al*, 2000; Figure 1). The A- and P-site tRNA substrates are positioned by base-pairing interactions between the conserved 3'-terminal CCA residues and conserved nucleotides in the A and P loops (Kim and Green, 1999), respectively. In general, site-directed mutagenesis of the inner shell of conserved nucleotides has little impact on peptidyl transfer activity (Polacek *et al*, 2001; Youngman *et al*, 2004). While initial structural studies focused particular attention on the nucleobase N3 position of A2451 and a potential role in acid-base catalysis (Muth *et al*, 2000; Nissen *et al*, 2000), only substitution of the 2'-OH of this residue appeared to substantially affect the efficiency of the reaction (Erlacher *et al*, 2005). These latter results were subsequently supported by molecular dynamic approaches that assign an important role to the 2'-OH of A2451 (Trobroy and Aqvist, 2005). These results, taken together with many earlier mutational analyses, suggest that the ribosome promotes catalysis of peptidyl transfer in large part through entropic fixation, consistent with biochemical experiments (Sievers *et al*, 2004).

By contrast, a role for the 2'-OH of the peptidyl-tRNA substrate seems increasingly likely, first, given its proximity to the α -amino nucleophile of the A-site substrate (Figure 1), and second, based on biochemical studies showing that this moiety is important for the catalysis of peptidyl transfer (Dorner *et al*, 2003; Weinger *et al*, 2004; Schmeing *et al*, 2005a) and peptide release (Brunelle *et al*, 2008). A role for the 2'-OH as a general base seems unlikely given (1) its intrinsically high pK_a , (2) the absence of an Mg^{2+} ion in its vicinity that could reduce its pK_a (Schmeing *et al*, 2005b), and (3) the absence of a corresponding pK_a in the pH dependence of the reaction (Bieling *et al*, 2006). Instead, the 2'-OH has been proposed to have an essential role in positioning substrates for catalysis and in a proton shuttle mechanism that moves protons in an active site well shielded from solvent (Dorner *et al*, 2002, 2003; Weinger *et al*, 2004; Trobroy and Aqvist, 2005; Schmeing *et al*, 2005a).

In the most recent version of a putative proton shuttle mechanism, the nucleophilic amino group donates a proton to the 2'-OH of A76, which spontaneously protonates the neighbouring 3'-OH, through a six-membered ring system (including substrate, ribosome, and critical water molecules;

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Figure 1 The environment of the active site of the ribosome during peptidyl transfer and peptide release. Structure of the PTC showing the proximity of the 2'-OH group of A76 to the nucleophile and leaving group and to that of A2451 during the course of peptide-bond formation (A) and RF-mediated peptide release (B). Models were constructed using PyMol and PDB files (2WDK, 2WDL, 1VQ7, 2WDG, 2WDI, 2X9R, 2X9S, 3D5A, and 3D5B).

Trobro and Aqvist, 2005). In this analysis, molecular dynamic simulations indicated that such a transition state could be further stabilized by the 2'-OH of A2451 donating a hydrogen bond to the 2'-OH of A76. This hydrogen-bonding network could provide a stable and pre-organized configuration that is well adapted to stabilizing the transition state. Such an active site might be expected to be relatively rigid and to sample few conformational states.

Despite its ability to rationalize most of the existing data, the proton shuttle mechanism has been called into question. In particular, Sprinzl and colleagues have argued that the 2'-OH of A76 of the peptidyl-tRNA is dispensable for peptidyl transfer (Koch *et al*, 2008). Using *in vitro* cell-free extracts, Sprinzl and colleagues showed that the ribosome can utilize a dA76 modified suppressor tRNA during protein synthesis to make functional full-length protein, albeit in decreased amount relative to the wild-type situation with rA76. Moreover, because the rate at which these proteins are synthesized was not reported, the quantitative contribution of the 2'-OH of the peptidyl-tRNA to catalysis in this multi-step reaction was difficult to evaluate. Indeed, earlier studies by the same group found that dA76-substituted tRNA^{Lys} failed to react in polyA-mRNA-directed synthesis of poly(Lys), while the very same tRNA was active in a single round of peptidyl transfer when tRNA was non-enzymatically loaded into the P site (Wagner *et al*, 1982); these data had been rationalized by arguing that the defects must lie in the translocation activities of the dA76 substrate (Wagner and Sprinzl, 1983).

Here, using a well-defined bacterial *in vitro* translational system, we re-explore the activity of dA76 tRNA substrates and reconcile these conflicting data. Our results indicate that in the absence of the 2'-OH group on the peptidyl-tRNA, the active site undergoes a slow rearrangement that renders it inaccessible to attack by the incoming aa-tRNA. We further find that the actual rate of peptidyl transfer with the dA76 peptidyl-tRNA is reduced by at least 100-fold relative to the wild-type rA76 substrate, at least 10-fold more than anti-

cipated based on the measured chemical reactivity differences of these two substrates. These findings provide an explanation for the documented complete loss in activity with the dA76-substituted peptidyl-tRNA when the ribosomal complexes were pelleted before the PT reaction (Weinger *et al*, 2004), and for the demonstrable (non-negligible) translation activity with dA76-substituted peptidyl-tRNA in an ongoing translation reaction (Koch *et al*, 2008). In conclusion, our data highlight the importance of the 2'-OH in peptidyl transfer catalysis where this functional group is essential for establishing an optimal and rigid network of hydrogen bonds within the active site.

Results

dA76 peptidyl-tRNA substrates are active as donors in peptidyl transfer reactions

Previous studies from our groups had shown that substitution of the 2'-OH of peptidyl-tRNA with either a hydrogen (dA76) or a fluorine (fA76) reduced the rate of peptidyl transfer on the ribosome by more than six orders of magnitude (Weinger *et al*, 2004). These experiments depended on an *in vitro* reconstituted translation system, where the many steps of translation can be isolated in order to evaluate their pre-steady state kinetic parameters. In direct conflict with these data, a recent report from the Sprinzl group found that the 2'-OH group of the peptidyl-tRNA to be dispensable for the peptidyl transfer reaction. In this latter study, a system was established where overall translation of particular mRNAs in an S30 extract could be directly monitored. More specifically, an amber codon (UAG) was substituted for an essential serine codon in the esterase 2 mRNA and the production of significant amounts of full-length functional protein was seen only in the presence of suppressor Ser-tRNA^{Ser(CUA)} (either rA76 or dA76) (Koch *et al*, 2008). While this system does not allow for the direct evaluation of effects on the actual rates of peptidyl transfer, the data seem inconsistent with the 10⁶-fold defect in catalysis reported by Weinger *et al* (2004).

Formally, these two contradictory reports could be reconciled in a number of different ways. First, because the S30 extract contains nucleotidyl transferase (CCA-adding enzyme) along with ATP and pyrophosphate, it seemed possible that the terminal dA76 of the dA76 suppressor Ser-tRNA^{Ser(CUA)} was exchanged in the reaction for rA76, though the group had provided some evidence that this was not the case. Alternatively, we wondered whether the hydroxyl group of the serine side chain might functionally substitute for the 2'-OH of A76, as we had observed for certain release factor variants (Shaw *et al*, submitted). Finally, the discrepancies might be explained by differences in the length of the peptidyl-tRNA moiety; in our assays, the ribosomes were programmed with short dipeptidyl-tRNA, whereas in the assays used by the Sprinzl group, the relevant step occurred on ribosomes containing a substantially longer peptidyl-tRNA.

The first possibility was tested using a ³²P-internally labelled tRNA^{Ser} prepared with either a terminal dA76 or rA76 using the CCA-adding enzyme (Nordin and Schimmel, 2002). The two tRNAs, dA76 and rA76, were incubated in an S30 extract and the identity of the terminal nucleotide analysed after the reaction by periodate cleavage sensitivity. Consistent with the reported results (Koch *et al*, 2008), the dA76 tRNA remained wholly resistant to periodate cleavage, suggesting that no significant exchange of the terminal dA76 had taken place in the extract (Supplementary Figure S1).

The second possibility was tested using our *in vitro* reconstituted bacterial translational system. For this experiment, we prepared ribosome initiation complexes (ICs) (loaded with f[³⁵S]-Met-tRNA^{fMet} in the P site and programmed with an mRNA encoding Met-Ser-Phe (MSF)) and reacted them all at once with two different ternary complexes for the Ser and Phe codons (either the rA76 or dA76 Ser-tRNA^{Ser} and Phe-tRNA^{Phe}) and elongation factor G (EFG). The formation of MSF tripeptide was then followed using our previously described electrophoretic TLC system (Youngman *et al*, 2004). As reported by Sprinzl and colleagues (Koch *et al*, 2008), the dA76 Ser-tRNA^{Ser} appeared to function as an active donor (i.e., when bound in the P site), albeit with a reduced yield (end point) relative to its rA76 counterpart (Supplementary Figure S2). We next looked at the activity of these same rA76 and dA76 Ser-tRNA^{Ser} species in ribosome complexes encoding Met-Ser-UAA (MSX); in this case, the stop codon in the A site is recognized by a class 1 release factor. In this case, we observed robust peptide release activity with either RF1 or RF2 with the rA76 Ser-tRNA^{Ser} species and no activity with the dA76 version (Supplementary Figure S2). These latter data are consistent with previous studies, indicating the 2'-OH of peptidyl-tRNA is essential for peptide release (Brunelle *et al*, 2008). Together, these data presented a conundrum—the potent activity of the dA76 Ser-tRNA^{Ser} in the peptidyl transfer reaction was consistent with the translation extract data from the Sprinzl group (Koch *et al*, 2008) (but not with data from the reconstituted system; Weinger *et al*, 2004) while the absence of release activity was consistent with data from the reconstituted system (Brunelle *et al*, 2008).

A step following translocation affects the reactivity of the dA76-substituted tRNA

We wondered whether discrepancies over the reactivity of dA76-substituted tRNAs could be attributed to differences in

the substrates used (Ser-tRNA^{Ser} versus Lys-tRNA^{Lys}) or to the way the reactions were set up. To test the first possibility, we performed the same basic experiment as above, but with rA76 and dA76 Lys-tRNA^{Lys} as in the earlier set of experiments (Weinger *et al*, 2004). As described earlier, ribosome ICs (programmed with Met-Lys-Phe, MKF) were reacted with two different ternary complexes (either the rA76 or dA76 Lys-tRNA^{Lys} and Phe-tRNA^{Phe}) and EFG, and tripeptide formation was measured. To our surprise, in agreement with the Ser-tRNA^{Ser} data above, and again in contrast with the earlier report (Weinger *et al*, 2004), the dA76-substituted tRNA^{Lys} was active as a donor in the P site for peptidyl transfer (as a Met-Lys-tRNA^{Lys}; Figure 2). As noted above for the dA76-substituted tRNA^{Ser} reaction, we again see end point defects that are indicative of compromised overall activity.

Another difference between the previously published experiments and those performed here was the timing of the various steps of translation, since to evaluate dA76 tRNAs in the P site, they must first be loaded in the A site, reacted with the P-site substrate (fMet-tRNA^{fMet} in this case), and translocated into the P site as a dipeptidyl-tRNA (e.g., fMet-Lys-tRNA^{Lys}). In the earlier study, stalled dipeptidyl-tRNA complexes were prepared and pelleted over a sucrose cushion following the translocation step; this protocol allowed us to specifically monitor the rate of peptide-bond formation or peptide release, independent of the rates of the preceding steps. Here, we added two sequential tRNAs simultaneously with EFG; in this case, the observed rate of tripeptide forma-

Figure 2 The donor activity of the dA76-substituted peptidyl-tRNA critically depends on the assay set-up. An autoradiograph of an electrophoretic TLC used to follow tripeptide formation with rA76 and dA76 tRNAs under different conditions. When a Met-Lys-Phe programmed IC (left panel) is incubated with rA76 Lys-tRNA^{Lys} and Phe-tRNA^{Phe} ternary complexes in the presence of EFG, efficient tripeptide synthesis is observed regardless of the course of ternary complexes addition. In contrast, for the dA76 reaction, no tripeptide synthesis is observed when Phe-tRNA^{Phe} ternary complex is added 5 min subsequent to the addition of the Lys-tRNA^{Lys} ternary complex and EFG; tripeptide synthesis is only observed when both ternary complexes and EFG are added simultaneously to the IC, albeit with a reduced yield (~40%). In agreement with these observations, a pelleted rA76 dipeptidyl RNC (right panel) reacts efficiently with the Phe-tRNA^{Phe}, while its dA76 counterpart fails to produce significant amount of the tripeptide.

tion or dipeptide release is a composite of several different steps in the pathway.

We asked whether these differences in protocol were responsible for the very different experimental outcomes. Indeed, when dipeptidyl-tRNA (either rA76 or dA76 containing) loaded ribosomal nascent-chain complexes (RNCs) are first pelleted and then reacted with the A-site substrate (Phe-tRNA^{Phe}), we see no peptidyl transfer activity with the dA76 tRNA^{Lys} substrate, in contrast to the rA76 reaction (Figures 2 and 3A). When the sequential substrates (Lys-tRNA^{Lys} and Phe-tRNA^{Phe}) are added simultaneously, we see that dA76-containing tRNA^{Lys} is quite reactive as a P-site donor. Alternatively, if Lys-tRNA^{Lys} ternary complex and EFG are added to the reaction and allowed to react for 5 min before the addition of Phe-tRNA^{Phe}, we see a dramatic loss in the donor activity of dA76-containing tRNA^{Lys} (Figure 2). We conclude that the dA76-containing dipeptidyl-tRNA ribosome complexes are inactivated over time, and completely so during the extended time frame of pelleting. A conformational change in the active site that is more readily permitted, or that is more stabilized in the absence of the 2'-OH group, would rationalize the observed behaviour.

Extracting rate constants for the putative conformational rearrangement and for peptidyl transfer

Although both the previously documented total loss of peptidyl transfer activity (Weinger *et al*, 2004) and the end point defects documented here with the dA76-substituted tRNA can be rationalized by an inactivating rearrangement step following translocation, our data nevertheless hint at a considerable slowing of the peptidyl transfer rate. Here, we used several approaches to disentangle the effects of the dA76 substitution on catalysis *per se* and on a potential conformational rearrangement of the catalytic centre. We first reasoned that if the acceptor activity of dA76 tRNA (i.e., functioning in the A site) was relatively less compromised than its donor activity (i.e., functioning in the P site), we could evaluate the defects in P-site function by directly incubating the previously described

ICs with two ternary complexes (either the rA76 or dA76 Lys-tRNA^{Lys} and Phe-tRNA^{Phe}) and EFG, and following the production of tripeptide (MKF) over time (as in Figure 3B). In this set-up, the disappearance of fMet signal reports on the first PT reaction (dipeptide formation, acceptor activity), while the appearance of fMet-Lys-Phe reports on the second PT reaction (tripeptide formation, donor activity). Similar to earlier studies (Zaher and Green, 2010a), for the rA76 substrate, the rate of the first PT reaction was $\sim 4\text{ s}^{-1}$ (Figure 3B), while that for the second PT reaction was $\sim 1\text{ s}^{-1}$, likely somewhat limited by the rate of translocation. For the dA76 substrate, the rate of the first PT reaction was ~ 400 -fold slower than that observed for rA76 (0.02 versus 4 s^{-1} , respectively). For the same dA76 substrate, the rate of tripeptide formation (reporting on the second PT reaction) was $\sim 0.016\text{ s}^{-1}$, with an end point of ~ 0.5 . We note that the apparent rate for the second PT reaction with dA76 Lys-tRNA^{Lys} closely matched the rate determined for the first PT reaction, suggesting that the second step might be limited by the first. As such, no meaningful evaluation of the P-site donor activity of the dA76-substituted tRNAs could be made. An explanation for the diminished acceptor activity of the dA76-substituted tRNA will be provided in a later section.

As an alternative approach to avoid the complications of the limiting acceptor activity of the dA76 substrate, we used the same MKF programmed ICs to generate fMet-Lys dipeptidyl RNCs paused in the pre-translocation state (by excluding EFG from the reaction) and subsequently reacted them with Phe-tRNA^{Phe} ternary complex in the presence of EFG. Because the rate of translocation is expected to be faster than the PT rate (at least with the dA76 substrate), the rate of tripeptide formation is in this case likely to report on the donor activity of the dA76 substrate. In this regime, the rate of tripeptide formation for the rA76 substrate was measured to be $\sim 1.5\text{ s}^{-1}$ (Figure 3C), somewhat slower than the 30 s^{-1} rate earlier determined for post-translocation RNCs with Phe-tRNA^{Phe} (Figure 3A), and hence likely limited by the somewhat slower rate of translocation and EFG dissociation. For

Figure 3 Disentangling the effects of the dA76 substitution of the peptidyl-tRNA on the rate of peptide-bond formation. (A) Time courses of tripeptide formation with a dipeptidyl Met-Lys RNC and Phe-tRNA^{Phe} ternary complex. Consistent with the rearrangement mechanism, the rate of peptide-bond formation with the dA76 dipeptidyl RNC could not be measured, while that with the rA76 complex was measured to be 30 s^{-1} . (B) Time courses of dipeptide and tripeptide formation with an IC and simultaneous incubation with Lys-tRNA^{Lys} and Phe-tRNA^{Phe} ternary complexes. The donor activity of the dA76-substituted peptidyl-tRNA was found to be limited by the acceptor activity of its aa-tRNA equivalent. The observed rate of Met-Lys-Phe tripeptide formation (dA-tri) is similar to that determined for the formation of the Met-Lys dipeptide (dA-di, $\sim 0.02\text{ s}^{-1}$), and is about 100-fold slower than that determined for the tripeptide with rA76 aa-tRNA (rA-tri). As seen earlier the yield of tripeptide formation in the presence of the dA76 substrate is $\sim 50\%$. (C) Time course of tripeptide formation between a pre-translocation dipeptidyl Met-Lys RNC and Phe-tRNA^{Phe} ternary complex in the presence of EFG. The rate of peptide-bond formation with a dA76-substituted peptidyl-tRNA is reduced by more than two orders of magnitude relative to the rA76 peptidyl-tRNA, while the end point was measured to be 0.4 instead of the 0.8 end point measured for the rA76 substrate.

the dA76 substrate, we measure a rate for tripeptide formation of 0.04 s^{-1} and an end point of 0.4 (down from the end point of 0.8 typically observed with rA76 substrate; Figures 2 and 3C). While we have not measured the rates of translocation directly, we have a lower limit estimation of the rate of translocation with the dA76-substituted tRNA (in experiments with a variant RF1 (GGS)), which allows us to conclude that the rates obtained here reflect the rate of PT (data not shown).

Due to the nature of the partitioning, the observed rate of tripeptide formation of 0.04 s^{-1} is the sum of the rates of PT and the putative rearrangement step, while the end point is equal to the fraction $k_{\text{PT}}/(k_{\text{PT}} + k_{\text{rearrangement}})$. From these equations and the data, we calculate rates of 0.016 and 0.024 s^{-1} for k_{PT} and $k_{\text{rearrangement}}$, respectively. These rates were broadly corroborated by an independent experiment where the end point of the reaction over time was estimated by puromycin reactivity (Supplementary Figure S3).

The dA76-substituted aminoacyl-tRNAs are chemically stabilized relative to their rA76 counterparts

While the measured rate of peptidyl transfer with the dA76-substituted tRNA is markedly slow relative to rA76 (0.016 versus 1.5 s^{-1} or 100-fold slower), it was important to determine if this difference is greater than might be anticipated based on inherent differences in the chemical reactivity of the substrates. In earlier experiments, we had failed to observe differences in the background hydrolysis reactivity of dA76 and rA76 versions of ($^{\text{N}}\text{Ac}$)₂-Lys-tRNA^{Lys} (Brunelle *et al*, 2008); other groups obtained similar results with different model substrates (Sievers *et al*, 2004). We had noted, however, that the observed rates were extremely slow, on the order of $0.05\text{--}0.07\text{ h}^{-1}$, suggesting that we had not identified the best assay for evaluating these differences. In order to more meaningfully evaluate the inherent reactivity of the substrates, we utilized less stable aa-tRNAs, rather than peptidyl-tRNAs, as substrates, and we looked at both hydrolysis and a faster aminolysis reaction. We began by looking at the rates of hydrolysis, and found the rate to be 20-fold faster (at pH 8.8) for rA76 Lys-tRNA^{Lys} than for the dA76 version (Figure 4), somewhat different from the earlier negligible differences measured at pH 7.4 (Brunelle *et al*, 2008). For the aminolysis reactions with the same substrates, the rA76 versions react 10–15-fold faster than the dA76 versions, irrespective of the nucleophile (Tris or hydroxylamine; Figure 4).

In the end, the ~ 100 -fold decrease in the rate of peptidyl transfer measured for the dA76-substituted tRNA is in part accounted for by the inherent ~ 10 -fold difference in chemical reactivity of the modified substrate, with the remaining ~ 10 -fold being attributed to the role of the 2'-OH in specifically facilitating catalysis on the ribosome.

Defects in the acceptor activity of dA76 aa-tRNA is due to loss of EFTu binding

As discussed earlier, the dA76-substituted tRNA has dramatically reduced A-site acceptor activity (Figure 3B). We were interested in determining whether the observed defect resulted from compromised interactions with EFTu or the ribosome. We first looked at acceptor activity for the rA76 and dA76 substrates in the presence and absence of EFTu. As expected, for the rA76 aa-tRNA, the addition of EFTu

Figure 4 The dA76-substituted aa-tRNA is inherently less reactive than its rA76 counterpart. A graph of the observed rates of background hydrolysis and aminolysis (with Tris and hydroxylamine) in a buffered solution. Points plotted represent rates measured from two independent measurements.

significantly increased the rate of PT from 0.02 to 3 s^{-1} ; by contrast, the addition of EFTu to the dA76 aa-tRNA had no discernible effect on the rate of PT (0.02 s^{-1}) (Figure 5A). These data raise the possibility that the dA76 aa-tRNA fails to bind or activate EFTu.

We tested this hypothesis by evaluating (1) EFTu-stimulated GTP hydrolysis and (2) binding to EFTu for both rA76 and dA76-substituted tRNA. To evaluate GTP hydrolysis activity, radioactively labelled ($[\gamma\text{-}^{32}\text{P}]\text{-GTP}$) ternary complex with rA76 and dA76 tRNAs were added to programmed ribosomes and the extent of GTP hydrolysis was visualized by TLC (Pape *et al*, 1998). The observed rate of GTP hydrolysis for the rA76 tRNA at 20°C was 10 s^{-1} , similar to previously reported values under similar conditions (Zaher and Green, 2010b), while there was no detectable GTP hydrolysis in the presence of the dA76 tRNA (Figure 5B). Next, we determined the binding parameters of the tRNAs to EFTu using a nuclease protection assay (Asahara and Uhlenbeck, 2002) that directly measures the off-rate of aa-tRNA from the protein. While the rA76 aa-tRNA dissociated from EFTu at a rate of 0.005 s^{-1} at 4°C , consistent with earlier reported values (Vorstenbosch *et al*, 2000), we could not measure a rate of dissociation for the dA76 aa-tRNA, as after only 5 s of incubation the aa-tRNA was fully degraded by RNase A (Figure 5C). These findings together suggest that the dA76-substituted aa-tRNA does not bind EFTu, thus explaining the substantial defects in acceptor activity.

dA76-substituted tRNA has no detectable donor activity in RF-mediated release

In light of our new understanding of the activities of the dA76-substituted tRNA in donor and acceptor roles on the ribosome during peptidyl transfer, we wanted to re-examine the activity of this tRNA as a donor for the peptide release reaction. ICs programmed with an MK(UAA) mRNA were incubated with a variety of release factors in the presence of either rA76 or dA76 Lys-tRNA^{Lys} ternary complex. As expected, with rA76 tRNA in the P site, quantitative peptide release of Met-Lys from the peptidyl-tRNA was observed with

Figure 5 Defects in the acceptor activity of the dA76 aa-tRNA are the result of poor ternary complex formation. (A) Time courses of dipeptide formation with an IC and Lys-tRNA^{Lys} tRNA. The aa-tRNA was added to a final concentration of 0.5 μ M (half of the IC concentration). For the rA aa-tRNA, the addition of EFTu stimulated the rate of PT by ~ 200 -fold (~ 0.02 versus 4 s⁻¹), while it had no discernible effect on the dA76 reaction (~ 0.02 s⁻¹ for both). (B) Time courses of GTP hydrolysis. While the rate of GTP hydrolysis for the rA76 was determined to be ~ 10 s⁻¹, no apparent rate could be measured for the dA76 substrate. (C) Time courses of aa-tRNA dissociation from EFTu.GTP using an RNaseA protection assay. While the rA76 aa-tRNA dissociated at a rate of ~ 0.05 s⁻¹, the dA76 aa-tRNA was fully dissociated from EFTu.GTP at the 5-s interval (the lowest time-point), suggesting that it never bound the factor or their interaction is very labile.

RF1 and RF2; in contrast, with dA76 tRNA in the P site, none of our class 1 release factor preparations (either over-expressed and purified RF1 and RF2, or endogenous RF2 carrying some important post-translational modifications) exhibited any release activity on the ribosome termination complex (Figure 6). While the rate of release is generally slower than that of PT (0.05 – 10 versus 1 – 150 s⁻¹, respectively) (Zaher and Green, 2009; Wohlgemuth *et al*, 2010); here, we explored release in a system where the observed rate with rA76 substrates is on the order of 10 s⁻¹ (with native methylated RF2; Zaher and Green, 2009), much faster than the putative conformational rearrangement that we have characterized. Therefore, we estimate a reduction in release activity of 10^5 -fold, 10 -fold of which can be attributed to inherent chemical differences, leaving 10^4 -fold for a contribution of the 2'-OH to release catalysis. At the simplest level, these findings broadly support the previous literature arguing that release factors depend critically on the 2'-OH of A76 for catalysis (Brunelle *et al*, 2008).

Discussion

The data presented here provide compelling evidence for a role for the 2'-OH of A76 of the peptidyl-tRNA in priming the active site of the ribosome for catalysis. In the absence of the 2'-OH group, we observe gradual loss of PT donor activity as a function of time, indicative of a conformational rearrangement that renders the peptidyl-tRNA inaccessible for attack by the incoming aa-tRNA. In support of this model, when the two aa-tRNAs that are required to complete the synthesis of tripeptide were added to the ICs in a concerted manner, efficient synthesis of the tripeptide was observed in the presence of a dA76-substituted P-site substrate; in contrast, when the same tRNAs were added sequentially, with or without an intermediate purification step, no synthesis of tripeptide was observed (Figure 2). Consistent with these ideas, we typically observed end point defects even in the concerted reaction, specifically with the dA76 substrate, a feature diagnostic of a discard process that competes with the PT reaction. These findings are consistent with a recent report showing demonstrable activity of the dA76-substituted substrate in an *in vitro* translation reaction (Koch *et al*, 2008). Moreover, these observations

Figure 6 RF-mediated release is inhibited in the absence of the 2'-OH of A76 of the peptidyl-tRNA. An autoradiograph of an electrophoretic TLC used to follow the release of the fMet-Lys dipeptide in the presence of the indicated Lys-tRNA^{Lys} and RF (oe and ce denote overexpressed and chromosomally expressed factors, respectively). As expected, when the depicted IC is incubated with rA76 Lys-tRNA^{Lys}, EFG, and any of the RFs, quantitative release of the dipeptide is observed in all cases. In contrast, when the rA76 aa-tRNA is substituted by the dA76 one, efficient release of the dipeptide is only observed in the presence of the rescuing RF1^{GGS} factor.

suggest that the 10^6 reduction in the rate of peptide-bond formation reported earlier by our groups (Weinger *et al*, 2004) was an overestimate of the actual defects of the dA76-substituted tRNA as a donor substrate.

Our model invoking a misorientation of dA76-substituted substrates in the active site is reminiscent of earlier ideas on the conformational flexibility of the active site. Early studies hinted at a pH-dependent conformational rearrangement in the active site (Muth *et al*, 2000; Bayfield *et al*, 2001; Katunin

et al, 2002), while more recent biochemical and structural studies documented 'induced fit' conformational rearrangements in the active site promoted on binding of the A-site substrate (Youngman *et al*, 2004; Schmeing *et al*, 2005b; Brunelle *et al*, 2006). Of particular relevance to the studies here is the fact that attempts by the Steitz group to solve structures of the large ribosomal subunit with bound dA76-substituted peptidyl-tRNA were unsuccessful (Schmeing *et al*, 2005a). The authors noted that under conditions where the rA76 mimic bound the P site, the dA76-substituted version instead bound the A site; only when sparsomycin was also added did the dA76-substituted tRNA bind in the P site. Hence, both biochemical and structural studies argue for an important role for the 2'-OH group in positioning the substrates for PT. The absence of the 2'-OH group is likely to result in the repositioning of either the carbonyl carbon of the leaving group or the amine group of the nucleophile, triggering the observed drastic reduction in the rate of peptide-bond formation (Weinger *et al*, 2004). What the structural studies could not predict, however, is the dynamic nature of the repositioning of the substrates in the absence of the 2'-OH group. The biochemical data presented here suggest that the rearrangement proceeds with a relatively slow rate of 0.02 s^{-1} after translocation is completed.

After disentangling the effects of the conformational rearrangement on the dA76-substituted substrate, we were able to conservatively estimate a reduction in PT catalysis of ~ 100 -fold, and think it is likely to be even greater (~ 2000 -fold, using the 30 s^{-1} rate obtained for rA76 in Figure 3A, when translocation does not limit PT). The overall 100-fold defect in donor activity can be partly explained by inherent reactivity differences for the rA76 and dA76 substrates that we measure to be about 10-fold (Figure 4). The remaining portion (a minimum of ~ 10 -fold, and more likely ~ 200 -fold) is a relatively modest value (certainly compared with the previous estimate of 10^6) (Weinger *et al*, 2004), suggesting a commensurate role in catalysis. A role for the 2'-OH in a hydrogen-bonding network that orients the substrate for catalysis may well explain the reduction in PT catalysis that we report, though we certainly cannot rule out a more significant role in facilitating proton transfer.

In addition to a role for the 2'-OH in the PT donor activity in the P site, we find an important role for this group in EFTu ternary complex formation; indeed, we failed to detect any binding between the dA76-substituted aa-tRNA and EFTu (Figure 5). These findings reconcile our initial observations of the inability of EFTu to stimulate the acceptor activity of the dA76 aa-tRNA (Figures 3B and 5A). These results are in direct conflict with earlier studies where no differences in the dissociation rates of the rA76 and dA76 aa-tRNAs from EFTu were observed (Pleiss and Uhlenbeck, 2001). The discrepancy between these earlier observations and our own might be reconciled by the different aa-tRNAs and EFTu factors utilized; Uhlenbeck and colleagues used *in vitro* transcribed variants of the yeast tRNA^{Phe} and *Thermus thermophilus* EFTu, while we used native tRNA^{Lys} and EFTu, both from *E. coli*.

Previously, our group also reported a critical role for the 2'-OH of the terminal adenosine of the peptidyl-tRNA during peptide release (Brunelle *et al*, 2008). Here, we re-examined peptide release to discern whether the reported substantial loss of activity could also be explained by the rearrangement

mechanism. Interestingly, unlike PT, both RF1- and RF2-mediated release were found to be fully dependent ($> 10^4$ -fold) on the presence of 2'-OH, where we observe no peptide release even when all the substrates were added in a concerted manner (Figure 6), that is, likely in the absence of the rearrangement step. The overall reactivity of the dA76-substituted peptidyl-tRNA ribosome complexes was established through the use of an RF1 variant carrying a serine substituted in the catalytically critical GGQ motif (Shaw *et al*, submitted); these data argue that the dA76-containing ribosome complexes are intact and in the anticipated reading frame. The stronger defects in RF catalysis that we observe for the dA76 substrate, relative to those measured for PT catalysis, may be indicative of mechanistic differences in the utilization of the 2'-OH group during the reaction pathways. Consistent with this proposal, many mutational studies have established distinct requirements for these reactions with respect to the identities of certain ribosomal residues (e.g., Polacek *et al*, 2003; Youngman *et al*, 2004).

In conclusion, our findings reconcile disagreement in the literature regarding the role of the 2'-OH of A76 of the peptidyl-tRNA in ribosome catalysis and provide new insights into the process by which the PTC of the ribosome can be modulated. The conformational rearrangement that occurs when the 2'-OH is omitted from the peptidyl-tRNA may be similar to those rearrangements that render the active site more active or less active during normal translation. There are numerous examples in the literature of various factors affecting the elongation rate, including particular peptide sequences in the exit channel (Tenson and Ehrenberg, 2002) or specific aminoacyl-tRNAs in the A or P sites (Berlinger, 2008). We should learn more about such complex regulation during translation as we figure out the very detailed molecular features of the active site.

Materials and methods

Reagents

70S tightly coupled ribosomes were purified from MRE600 (ATCC29417) using previously published protocols (Moazed and Noller, 1986). IF2, EFTu, EFG, RF1, RF2, Phenylalanine Synthetase (PheRS), Lysine Synthetase (LysRS), and peptidyl hydrolase (Pth) were purified as His-tagged factors using Ni-NTA chromatography as described earlier (Zaher and Green, 2009). For EFTu and EFG, the His-tag was removed using His-tagged TEV protease and passed second time over Ni-NTA to collect the flowthrough. Purification of His-tagged nucleotidyl transferase (CCA-adding enzyme) was performed as described earlier (Weinger *et al*, 2004). Native IF1 and IF3 were overexpressed and purified as described elsewhere (Brunelle *et al*, 2006). *E. coli* tRNA^{Met}, tRNA^{Phe}, and tRNA^{Lys} were purchased from Chemical Block (Moscow, Russia). mRNA sequences were transcribed from double-stranded DNA templates using *in vitro* transcription by T7 RNA polymerase (Zaher and Unrau, 2004). Exchange of the terminal adenosine of tRNA^{Lys} for rA or dA nucleotide was carried out as described (Nordin and Schimmel, 2002). Briefly, tRNA^{Lys} at $20\text{ }\mu\text{M}$ was incubated with $1\text{ }\mu\text{M}$ CCA-adding enzyme at 37°C in 50 mM Tris pH 7.5, 20 mM MgCl₂, 1 mM pyrophosphate, 0.5 mM DTT, and 8 mM ATP or dATP to obtain rA76 and dA76 tRNA^{Lys}, respectively. The dA76 tRNA was further treated with 10 mM sodium periodate to remove possible rA contamination before phenol, chloroform extraction, which was followed by ethanol precipitation. Aminoacylation of tRNAs was carried out as described earlier (Zaher and Green, 2009).

Ribosome complex formation

ICs were prepared by incubating $2\text{ }\mu\text{M}$ 70S ribosomes with IF1, IF2, IF3, [³⁵S]-Met-tRNA^{Met} ($3\text{ }\mu\text{M}$ each), mRNA ($6\text{ }\mu\text{M}$), and GTP

(2 mM) in polymix buffer (Jelenc and Kurland, 1979) (95 mM KCl, 5 mM NH₄Cl, 5 mM Mg(OAc)₂, 0.5 mM CaCl₂, 8 mM putrescine, 1 mM spermidine, 5 mM potassium phosphate pH 7.5, 1 mM DTT) at 37°C for 45 min. Dipeptidyl ribosomal nascent chains (RNCs) were generated by incubating equivalent volumes of ICs (1 μM final concentration) with elongation mixture containing 30 μM EFTu, 1 μM Lys-tRNA^{Lys}, 3 μM EFG, and 2 mM GTP in polymix buffer at 37°C for 5 min. Both the ICs and RNCs were purified away from excess factors by pelleting over a sucrose cushion in a TLA100.3 rotor (Beckman) at 69000 r.p.m. for 2 h, and resuspended in polymix buffer.

Donor peptidyl transferase assays

Reactions involving purified RNCs were conducted by initially incubating EFTu (30 μM) with GTP (2 mM) for 15 min followed by the addition of Phe-tRNA^{Phe} (3 μM), and incubated for an additional 15 min. For the rA76 complexes, PT was initiated by rapidly mixing the resulting ternary complex with equal volume of RNC (1 μM final concentration) on a quench-flow instrument (RQF-3 quench-flow, KinTek Corporation) at 37°C. The reaction was quenched by the addition of KOH to a final concentration of 0.5 M. RNCs carrying the dA76-substituted peptidyl-tRNA were reacted with the ternary complex on the bench due to their slow reactivities. The tripeptide products were resolved from unreacted dipeptides using cellulose TLCs that were electrophoresed in pyridine acetate buffer pH 2.7 (Youngman *et al*, 2004).

Reactions involving concordant addition of ternary complexes were performed by adding Lys-tRNA^{Lys} (A or dA, 1 μM) ternary complex and EFG (3 μM) to the Phe-tRNA^{Phe} ternary complex mixture described above. The mixture was then added to ICs (1 μM final concentration) to initiate the PT reactions. Time-points were taken and the products resolved as described earlier. The disappearance of the f[³⁵S]-Met signal on the TLCs reported on the acceptor activity of the Lys-tRNA^{Lys} aa-tRNA, while the appearance of the fMet-Lys-Phe tripeptide reported on the donor activity of the peptidyl-tRNA.

Reactions involving sequential addition of ternary complexes were carried out by forming two ternary complexes: Lys-tRNA^{Lys} (A or dA), which also contained EFG at 3 μM; and Phe-tRNA^{Phe}. ICs were first incubated with Lys-tRNA^{Lys} ternary complex for 5 min before the addition of the Phe-tRNA^{Phe}.

Reactions involving pre-translocation complex were carried out by forming two ternary complexes analogous to the one described above, except that EFG was added to the Phe-tRNA^{Phe} ternary complex instead of the Lys-tRNA^{Lys} one. Dipeptide formation was initiated by incubating the ICs with the Lys-tRNA^{Lys} ternary complex for 5 min, tripeptide formation was monitored by adding Phe-tRNA^{Phe} and EFG to the pre-translocation (A/P hybrid state) dipeptide complex. At each time-point, two aliquots were taken: one aliquot was quenched with KOH to observe tripeptide formation as a function of time, while the other was quickly reacted with peptidyl hydrolase before quenching in 2% formic acid to correct for A/P peptidyl-tRNA drop-off (Dorner *et al*, 2006).

aa-tRNA assays

The non-enzymatic acceptor activity of rA76 and dA76 of Lys-tRNA^{Lys} was simply determined by incubating ICs (1 μM) with the corresponding aa-tRNA (0.5 μM). GTP hydrolysis assays were

conducted by first generating ternary complex containing EFTu (4 μM), Lys-tRNA^{Lys} (5 μM), and [γ -³²P]-GTP (1 μM) without further purification. The ternary complex was rapidly mixed with ICs on the quench-flow apparatus and the reaction was stopped by the addition of 25% formic acid. Inorganic phosphate was resolved from unreacted GTP using PEI cellulose thin-layer chromatography with 0.5 M KH₂PO₄ pH 3.5 as a mobile phase. Dissociation of the aa-tRNA from EFTu.GTP at 4°C was performed using an RNase A protection assay (Asahara and Uhlenbeck, 2002). Briefly, ternary complex was generated by incubating rA76 or dA76 [¹⁴C]-Lys-tRNA^{Lys} (~1 μM) with EFTu (20 μM) and GTP (2 mM) at 37°C for 15 min; the mixture was then moved to 4°C before RNase A was added to a final concentration of 50 ng μl⁻¹. Aliquots were taken as a function of time and quenched with ice-cold 10% TCA solution containing 0.1 mg ml⁻¹ carrier tRNA. Samples were then applied onto filter papers, and washed extensively with 5% TCA followed by ethanol using a vacuum manifold before counting on a scintillation counter to determine the fraction of tRNA protected from the RNase by EFTu. The rate of background hydrolysis/aminolysis of the rA76 and dA76 Lys-tRNA^{Lys} was determined by incubating the [¹⁴C]aa-tRNAs (~1 μM) in 50 mM Tris pH 8.8 at 37°C. Aliquots were taken at various times and quenched with 2% formic acid. Under these conditions, two products were observed: a slower moving one that migrates to the same spot as the Lys amino acid on the electrophoretic TLC, that is, corresponding to the hydrolysis reaction; and a faster moving product corresponding to the aminolysis (trisolysis) reaction. The rate of aminolysis with the hydroxylamine (HA) nucleophile was determined by adding HA to the aa-tRNAs to a final concentration of 2 M at pH 6.8.

Release assays

ICs programmed with an mRNA encoding Met-Lys-STOP (unless otherwise stated) were incubated with EFTu.Lys-tRNA^{Lys}.GTP ternary complex, EFG, and release factor (RF1 or overexpressed RF2 or chromosomally expressed RF2 or RF1^{GGS} variant). Aliquots were taken at various times and quenched with 2% formic acid and resolved using the electrophoretic TLC system described earlier.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: All of the authors designed the experiments and wrote the manuscript, HSZ and JJS performed the experiments.

Conflict of interest

The authors declare that they have no conflict of interest.

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