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Deacylated tRNA is released from the E site upon A site occupation but before GTP is hydrolyzed by EF-Tu

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ABSTRACT

The presence or absence of deacylated tRNA at the E site sharply influences the activation energy required for binding of a ternary complex to the ribosomal A site indicating the different conformations that the E-tRNA imparts on the ribosome. Here we address two questions: (i) whether or not peptidyltransferase—the essential catalytic activity of the large ribosomal subunit—also depends on the occupancy state of the E site and (ii) at what stage the E-tRNA is released during an elongation cycle. Kinetics of the puromycin reaction on various functional states of the ribosome indicate that the A-site substrate of the peptidyltransferase center, puromycin, requires the same activation energy for peptide-bond formation under all conditions tested. We further demonstrate that deacylated tRNA is released from the E site by binding a ternary complex aminoacyl-tRNA•EF-Tu•GDPNP to the A site. This observation indicates that the E-tRNA is released after the decoding step but before both GTP hydrolysis by EF-Tu and accommodation of the A-tRNA. Collectively these results reveal that the reciprocal linkage between the E and A sites affects the decoding center on the 30S subunit, but does not influence the rate of peptide-bond formation at the active center of the 50S subunit.

INTRODUCTION

The ribosomal elongation cycle consists of three basic reactions, namely the binding of the new aminoacyl-tRNA (aa-tRNA) according to the codon presented at the decoding center (reaction 1 of Figure 1A), the peptide-bond formation (reaction 2) and the translocation reaction (reaction 3). This cycle, described within the framework of the allosteric three-site model (1), is depicted in Figure 1A. According to this model, there are at least two tRNAs on the ribosome during the elongation cycle: tRNAs in the A and P sites prior to translocation (PRE state) and tRNAs in the P and E sites following translocation (POST state) as seen in Figure 1A. The allosteric three-site model also states that A-site occupation triggers the release of the deacylated tRNA from the E site (2,3). The linkage between A and E sites is also indicated by the fact that the activation energy is significantly larger with an occupied E site than with a free one, 120 and 80 kJ/mol, respectively (4). However, the exact step of A site binding that triggers release of the E-site tRNA has not yet been identified. Three possible steps are illustrated in Figure 1A. (i) The PRE state (1b), where the A-tRNA is still bound in the form of the ternary complex and makes codon–anticodon interaction with the A site codon of the mRNA. (ii) The PRE state (1c), where the aminoacyl moiety at the CCA end of the A-tRNA is accommodated into the A site at the peptidyltransferase center (PTC) on the 50S subunit. This step requires GTP hydrolysis and release of EF-Tu. (iii) Finally, the PRE state (2) arises following peptide-bond formation, such that the peptidyl moiety is now attached to the A-tRNA and the P-tRNA is now deacylated (uncharged). Release of the E-tRNA could be triggered at any one of these steps.

There are many studies supporting an allosteric linkage between the A and E sites. For example, (i) the antibiotic edeine, which binds in the E site on the 30S subunit induces translational misreading at the A site (3), (ii) weakening of the E-tRNA via mutations at the S7–S11 interface, which binds the anticodon loop of an E-tRNA, induces dramatic selection problems such as misincorporation and readthrough (5), and (iii) loss of codon–anticodon interaction at the E site provokes high-efficiency frameshifting (6,7). Furthermore, allosteric effects between the A and E sites have been observed in the crystal structure of 70S ribosomes from *Thermus thermophilus* at 4.5 Å, where components of the E site were well ordered when an E-tRNA was present, whereas the elements

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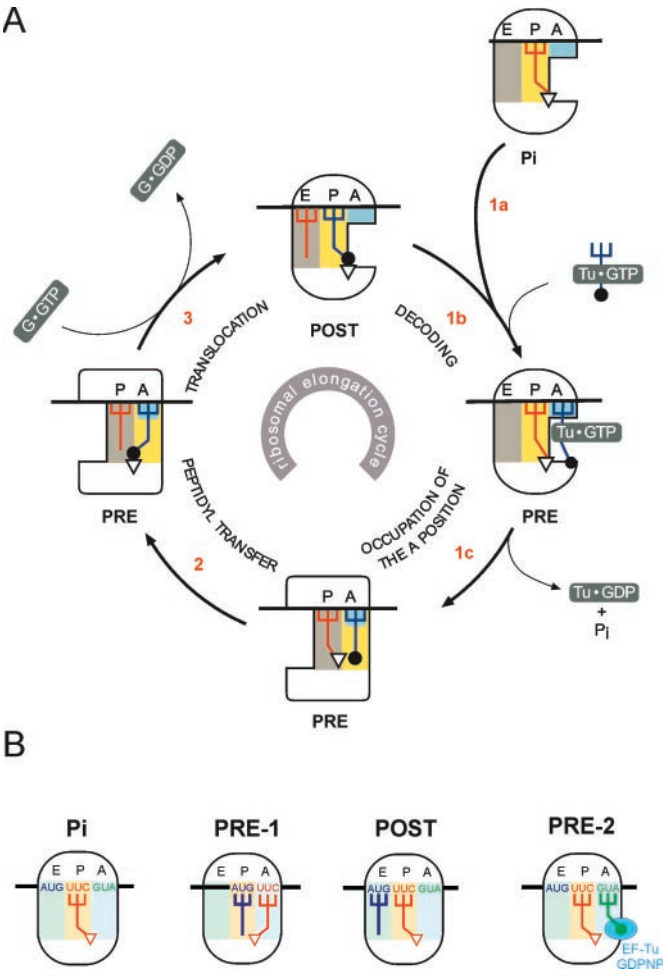

Figure 1. (**A**) The elongation cycle in the frame of the allosteric three-site model. The elongation cycle consists of three basic reactions, viz. the binding of the new aa-tRNA at the decoding center (reaction 1), peptide-bond formation (reaction 2) and translocation reaction (reaction 3). The reciprocal linkage between A and E sites is indicated by a gap: an occupied E site induces a low-affinity A site and vice versa. A-site occupation induces the release of the E-tRNA [for review see (1)]. (**B**) Ribosomal complexes in different functional states prepared in this work. For explanations see text.

were disordered when the A site instead of the E site was occupied (8). Since the PTC is part of A and P sites and antibiotics that bind at the PTC on the 50S subunit have been shown to affect tRNA decoding at the A site (9), we rationalized that peptide-bond formation could also be regulated by the functional state of the ribosome, specifically the presence or absence of an E-tRNA.

Here we have determined the step of A-site occupation at which the E-tRNA is released from the ribosome, revealing that the E-tRNA is released before both EF-Tu-dependent GTP hydrolysis and accommodation of the aa-tRNA into the A site. In addition, we show that the rate of transfer of peptidyl-moiety to puromycin is the same for Pi and POST states, revealing for the first time that peptide-bond formation is not regulated during protein synthesis. These results suggest that the occupation state of the E site influences only the 30S component of the A site, i.e. the decoding center, without effecting the 50S component of the A site, namely, the PTC.

MATERIALS AND METHODS

Reassociated 70S ribosomes free of endogenous tRNAs and mRNAs were prepared according to (10). Heteropolymeric mRNAs with two (MF-mRNA) or three (MVF-mRNA) unique codons in the middle were prepared with run-off transcription, according to (11). *N*-acetyl-[^{14}C]Phe-tRNA (Ac[^{14}C]-Phe-tRNA), *N*-acetyl-[^{14}C]Val-tRNA (Ac[^{14}C]Val-tRNA), [^{3}H]Val-tRNA, EF-Tu and EF-G were prepared as described previously (3).

All complexes were prepared in polyamine buffer system which contained 20 mM HEPES–KOH (pH 7.5 at 0°C), 4.5 mM $MgAc_2$, 4 mM 2-mercaptoethanol, 150 mM NH_4Ac, 0.05 mM spermine and 2 mM spermidine. This buffer approximates physiological conditions, with respect to the concentrations of the essential ions using NH_4^+ instead of K^+.

Construction of the Pi complexes. 70S ribosomes (0.3 μM) were incubated with mRNA (MF-mRNA, or MFV-mRNA, molar ratio mRNA:70S = 7:1) plus AcPhe-tRNA or AcVal-tRNA (molar ratio 1.5:1), at 37°C for 30 min in a volume of ~1 ml. Under these conditions, >85% of the ribosomes carried a peptidyl-tRNA analog at the P site, and had free A and E sites.

Construction of the PRE-1 complexes. 70S ribosomes programmed with MF-mRNA (described above) were incubated for 15 min at 37°C with uncharged $tRNA_f^{Met}$ (molar ratio to ribosomes 1.5:1), in order to prefill the P site. Subsequently, AcPhe-tRNA was added (molar ratio 1.5:1) and incubated for an additional 30 min at 37°C to allow non-enzymatic A-site binding. The puromycin reaction yielded no product indicating that $tRNA_f^{Met}$ was at the P site and AcPhe-tRNA was bound at the A site.

Construction of the POST complexes. PRE complexes (prepared as described above) were translocated in the presence of EF-G (molar ratio to 70S 0.2:1) and GTP (0.5 mM) during an incubation at 37°C for 10 min. According to the puromycin reaction at least 80% of the acylated tRNA was translocated to the P site.

Construction of the PRE-2 state complexes. POST state complexes carrying a $tRNA_f^{Met}$ (labeled with ^{32}P when indicated) in the E site and an Ac[^{14}C]Phe-tRNA in the P site were incubated with the ternary complex [^{3}H]Val-tRNA•GTP•EF-Tu at 25°C for 2 min (molar ratio 1.6:1). Under these conditions, a peptide bond is formed and the peptidyl-tRNA is exclusively bound at the A site, as demonstrated with the puromycin reaction. In another series of experiments, we used GDPNP instead of GTP in order to freeze the ribosomal complex in the PRE state prior to peptide-bond formation.

For kinetics of the puromycin reaction, ribosomal complexes were freed of factors, excess of substrates and GTP, via centrifugation through a 10% sucrose cushion in the same buffer (Beckman rotor TL-100, 45 000 *g* for 18 h, 4°C).

In each case, the ribosomal bound tRNAs were measured by nitrocellulose filtration, and the site location of the acylated tRNA was identified by the puromycin reaction.

Kinetics of the puromycin reaction

After formation of the ribosomal complexes, puromycin was added at the indicated concentrations (or if not indicated at 1 mM) and the reaction was carried out at various temperatures

in a volume of 180 µl. At various time intervals an aliquot of 20 µl was withdrawn and the reaction stopped by adding an equal volume of 0.3 M sodium acetate (pH 5.5) saturated with $MgSO_4$. The incubation mixture was extracted with 1 ml ethyl acetate and the radioactivity in 800 µl of the organic phase was measured after mixing with 5 ml scintillation liquid. Background controls (minus puromycin) were subtracted. The puromycin product was expressed as percentage of the ribosomal complex formed before adding puromycin (measured by nitrocellulose filtration). The puromycin reaction was employed for two distinct purposes: (i) to measure the activity of peptidyl transferase via determination of k_{cat} and K_M and (ii) to determine the amount of ribosomal complex that is puromycin reactive. In the first case the complete time course of the reaction was measured, while in the second only the extent of the reaction was required.

Data processing

If peptide-bond formation between the acyl residue of the tRNA at the P site and puromycin can be described as a first-order reaction, the following integrated rate law applies

$$\ln\left[\frac{C_0}{C_0 - \mathrm{P}}\right] = k_{obs} \cdot t, \quad \mathbf{1}$$

where C_0 is the initial concentration of the tRNA•ribosome complex reactive with puromycin (S) and k_{obs} is the observed rate constant of peptide-bond formation. As shown in the Results section the reaction indeed followed a first-order law. Then the relationship between the concentration of puromycin and the apparent rate constant k_{obs} is given by the equation

$$k_{obs} = \frac{k_{cat}\mathrm{S}}{K_S + \mathrm{S}}. \quad \mathbf{2}$$

where K_S is the dissociation constant of the encounter complex between tRNA•ribosome•mRNA complex and puromycin.

The apparent rate constant k_{obs} was measured for several functional ribosomal complexes at various temperatures, in a range of puromycin concentrations and with different donors. From the double reciprocal plot $1/k_{obs}$ versus 1/[puromycin], we calculated both k_{cat} and K_S (Figure 3B).

The apparent rate constants observed at various temperatures from 0 to 37°C for both the Pi and the POST complexes were processed in an Arrhenius plot according to

$$\ln k_{obs} = \ln A - \frac{E_a}{RT}, \quad \mathbf{3}$$

where E_a is the Arrhenius activation energy which can be estimated from the slope of the resulting regression line in the graph $\ln/k_{obs}$ versus $1/T$ and equals $-E_a/R$. The free enthalpy of activation $\Delta H^{\neq}$ was calculated according to

$$\Delta H^{\neq} = E_a - RT \quad \mathbf{4}$$

with R the gas constant and T the temperature in degree Kelvin. The free energy of activation $\Delta G^{\neq}$ is given by the Eyring equation

$$\Delta G^{\neq} = -2.3RT \log\left(\left[\frac{k_{cat}}{K_S}\right]\frac{Nh}{RT}\right), \quad \mathbf{5}$$

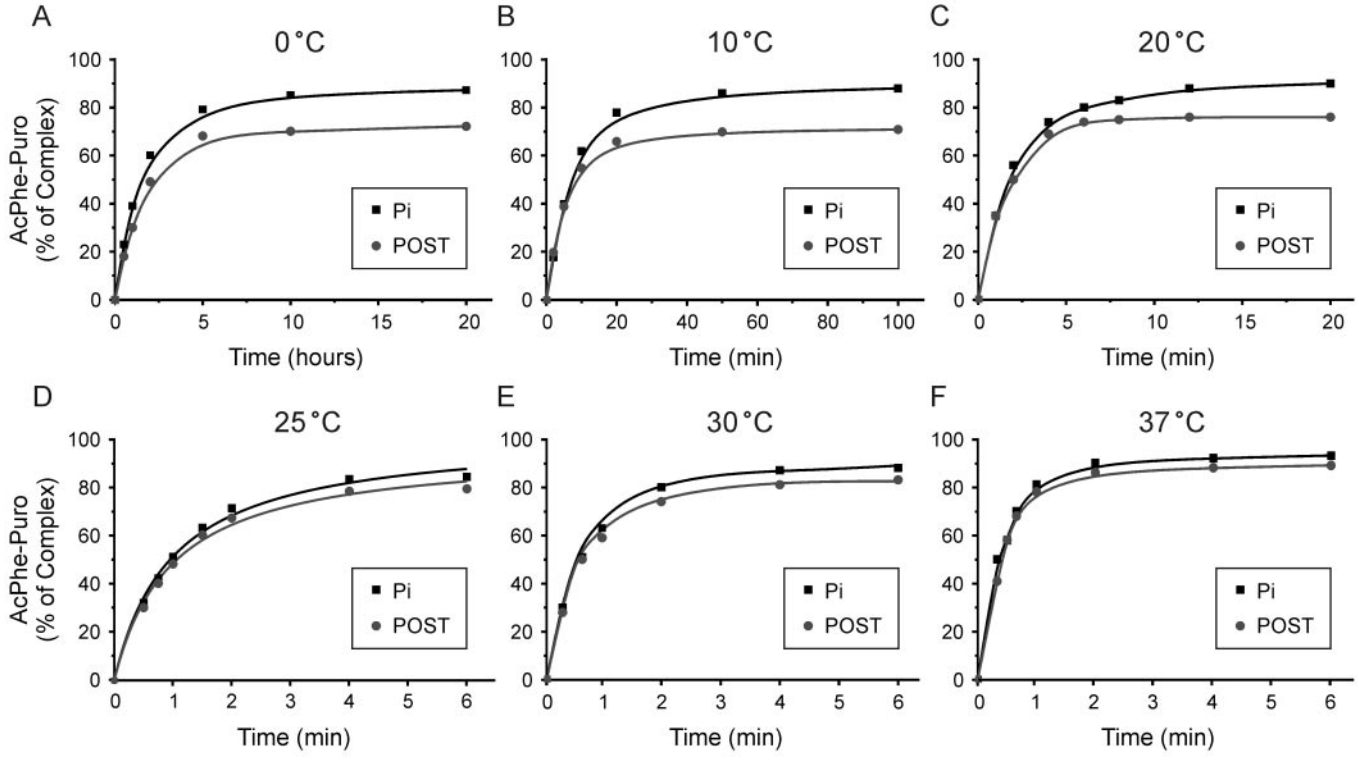

Figure 2. Kinetics of the puromycin reaction, at the following temperatures: 0, 10, 20, 25, 30 and 37°C. The puromycin concentration was 1 mM and the product AcPhe-puromycin was expressed as a percentage of the preformed ribosomal complex (Pi and POST, squares and circles, respectively) as measured via nitrocellulose filtration. Note the different time scales.

where h and N are the Planck's and Avogadro's constants, respectively, and $T\Delta S^{\neq}$ by

$$T\Delta S^{\neq} = \Delta H^{\neq} - \Delta G^{\neq}, \quad \mathbf{6}$$

where $\Delta S^{\neq}$ is the entropy of activation.

RESULTS AND DISCUSSION

We determined kinetic and thermodynamic parameters of the puromycin reaction performed with three ribosomal complexes, (i) Pi, (ii) POST and (iii) a special PRE complex (PRE-2), where the ternary complex is frozen in the A site by the non-hydrolyzable GTP analog GDPNP (Figure 1B). The Pi state is similar to a 70S initiation complex (i for initiation) in that it contains an empty E site and carries an AcPhe-tRNA at the P site. The POST complex is a standard complex of the elongation cycle, representing a posttranslocational ribosome state by having deacylated $tRNA_f^{Met}$ at the E site and an AcPhe-tRNA, the peptidyl-tRNA mimic, at the P site. The occupation of the tRNAs in the various states are compiled in Table 1. The occupancy of the P site in Pi (AcPhe-tRNA) and PRE-1 state ($tRNA_f^{Met}$) was ~85% and binding of AcPhe-tRNA to the A site showed that 74% of ribosomes carried AcPhe-tRNA in the A site of the PRE state ribosomes. Following translocation (addition of EF-G and GTP), the occupancies of the tRNAs in the POST complex remained the same, except that the $tRNA_f^{Met}$ was located at the E site and the AcPhe-tRNA was at the P site. This demonstrates that the tRNA is stably bound at the E site following translocation under these buffer conditions. This contrasts with the loss of E-tRNA found to occur following translocation in standard buffers without polyamines as well as in polymix buffer (12). It should be noted that all complexes were isolated through a sucrose cushion via an 18 h centrifugation to remove elongation factors, unbound tRNAs and GTP (Materials and Methods), thus reiterating the stable binding of the $tRNA_f^{Met}$ at the E site. This stability is consistent with the presence of E-tRNA in native polysomes following an isolation procedure of several hours (13).

Using these complexes we wanted to check whether the peptidyltransferase activity is regulated by the functional state of the ribosome. In order to do this the kinetics of the puromycin reaction were performed with Pi and POST complexes at different temperatures, ranging from 0 to 37°C (Figure 2). As expected, the rate of transfer of the AcPhe moiety of the P site bound tRNA to puromycin, forming AcPhe-Puro, slows as the reaction temperature is decreased. Furthermore, the data for Pi and POST complexes were almost identical at all the temperatures measured, providing the first hint that the peptidyltransferase activity is not regulated by the ribosome functional state, nor in this case the presence of an E-tRNA.

If we assume that the equilibrium of puromycin binding to ribosomes is fast and the subsequent peptide-bond formation between the acyl residue of the P-tRNA and puromycin is slow and rate-limiting, then the kinetics of the puromycin reaction

$$C + S \overset{K_S}{\rightleftharpoons} CS \xrightarrow{k_{cat}} P + C'$$

should follow a first-order law. In the scheme, the left half represents the binding reaction where C is the tRNA•ribosome complex and S is puromycin, and the right half, peptide-bond formation with k_{cat} as the rate-limiting step and P is the product acyl-puromycin. If the data from Figure 2 are plotted according to the first-order rate law (Materials and Methods), then a linear relationship is observed in all cases (Figure 3A), which verifies the rate law assumption.

Next, we determined k_{cat} and K_S. To this end we measured the relationship between the puromycin (S) concentration and

Table 1. Occupation of tRNAs in various ribosome functional states using MFV-mRNA[a]

Complex[b]	$[^{32}P]tRNA_f^{Met}$	Ac[^{14}C]Phe-tRNA	[^{3}H]Val-tRNA
Pi		0.85	
PRE-1	0.87	0.74	
POST	0.85	0.75	
PRE-2	0.15	0.65	0.60

[a]All values are presented as ν (pmoles bound isotopes per pmol 70S ribosomes).
[b]All complexes were measured after isolation through sucrose cushion to remove unbound elongation factors, unbound tRNAs and GTP.

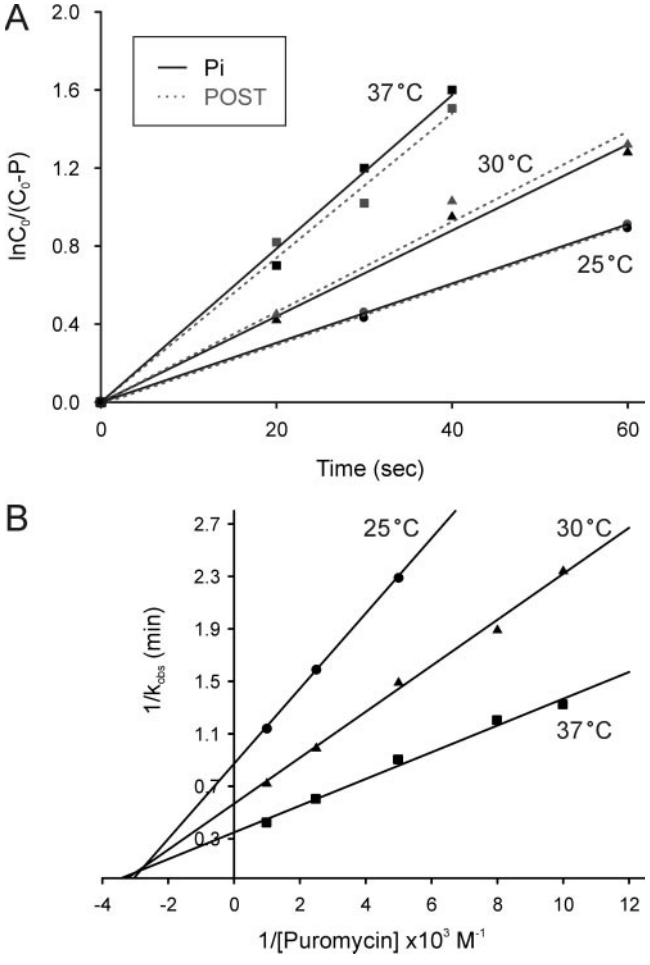

Figure 3. (**A**) First-order fitting of the data of Figure 2 at the temperatures of 25 (circles), 30 (triangles) and 37°C (squares) for Pi and POST complexes. The slope of the plots gives the apparent rate constant of product formation (k_{obs}) at the specified puromycin concentration (1 mM). (**B**) Double reciprocal plot of puromycin reactions at temperatures 25 (circles), 30 (triangles) and 37°C (squares). The intercept on the y-axis gives $1/k_{cat}$, while the intercept on the x-axis gives $1/K_S$.

Table 2. The catalytic activity of peptidyltransferase at different temperatures for the three different functional ribosomal states; Pi, POST and PRE-2[a]

Temperature (°C)	k_{cat}/K_S (M^{-1} s^{-1}) Pi	POST	PRE-2
0	1.6 ± 0.2	1.5 ± 0.2	1.4 ± 0.2
10	8.0 ± 1.0	8.5 ± 1.0	8.5 ± 1.0
20	36 ± 2	35 ± 2	34 ± 2
25	55 ± 3 50 ± 5[b]	54 ± 3	52 ± 3
30	82 ± 5	80 ± 5	78 ± 5
37	140 ± 5	138 ± 6	135 ± 5

[a]The activity is expressed by the ratio k_{cat}/K_S, which were calculated from the double reciprocal plots like Figure 3B.
[b]Ac[^{14}C]Val-tRNA was used instead of Ac[^{14}C]Phe-tRNA.

the apparent rate constant k_{obs} according to Equation 2 given in Material and Methods. The apparent rate constant k_{obs} was measured for Pi and POST complexes at various temperatures in a range of puromycin concentrations. The data were processed in a double reciprocal plot of $1/k_{obs}$ versus 1/[S] and representative plots are given in Figure 3B that were virtually identical for Pi and POST. The values of K_S and k_{cat} were estimated from the intercepts with x-axis and y-axis, respectively. k_{cat} values are varied as a function of temperature (Figure 3B), whereas K_S values were kept constant and equal to $4 \times 10^{-4} \pm 0.5 \times 10^{-4}$ M. The ratio k_{cat}/K_S, which expresses the activity status of peptidyltransferase, was calculated for each complex at different temperature and the values are summarized in Table 2. In addition, two controls were included: first, we checked whether the puromycin reaction of the Pi state was affected by a preceding incubation with EF-G•GTP, to enable a better comparison with the POST state that is formed by incubating with EF-G and GTP. Except for a spurious increase in the extent of reaction observed in some experiments, no significant change in the kinetic parameters was observed (data not shown). Second, the donor of the puromycin reaction on the ribosome was changed: instead of AcPhe-tRNA, Ac[^{14}C]Val-tRNA was used for P site binding in the presence of MFV-mRNA (Pi state). The kinetics of the puromycin reaction was performed at 25°C and the rate of reaction was shown to be independent of the species of donor occupying the P site (Table 2).

Some thermodynamic parameters were also derived from the kinetic data. The observed rate constants (k_{obs}) determined at various temperatures were used to determine the activation energy E_a by applying the Arrhenius equation (Equation 3 in Materials and Methods), which enables the activation parameters of free energy $\Delta G^{\neq}$, of enthalpy $\Delta H^{\neq}$ and entropy $\Delta S^{\neq}$ to be calculated using Equations 4–6 in Materials and Methods. The obtained data are listed in Table 3. The calculated values of $\Delta G^{\neq}$, $\Delta S^{\neq}$ and $T\Delta S^{\neq}$ are comparable with those reported previously [Ref. (14) and erratum], despite the fact that Sievers *et al*. used a different substrate, namely, fMet-Phe-tRNA instead of AcPhe-tRNA.

Next we analyzed whether the E-tRNA is released before EF-Tu-dependent GTP hydrolysis. Previously, we have shown that the A-site occupation with the ternary complex Val-tRNA•EF-Tu•GTP induced an almost quantitative release of the E-[^{32}P]tRNA (3), i.e. the deacylated tRNA was released from the E site at some point during decoding and accommodation of the aa-tRNA at the A site, however, which of the

Table 3. Activation parameters for catalyzed and uncatalyzed formation

Parameter (at 25°C)	AcPhe-Puro (catalyzed)	Uncatalyzed reaction[a]
k_{cat}/K_S ($M^{-1}s^{-1}$)	55	3×10^{-4}
E_a (Kcal/mol)	19.1	9.7
$\Delta H^{\neq}$(Kcal/mol)	18.5	9.1
$\Delta G^{\neq}$(Kcal/mol)	15.1	22.2
$T\Delta S^{\neq}$(Kcal/mol)	3.4	−13.1

[a]From Ref. (14).

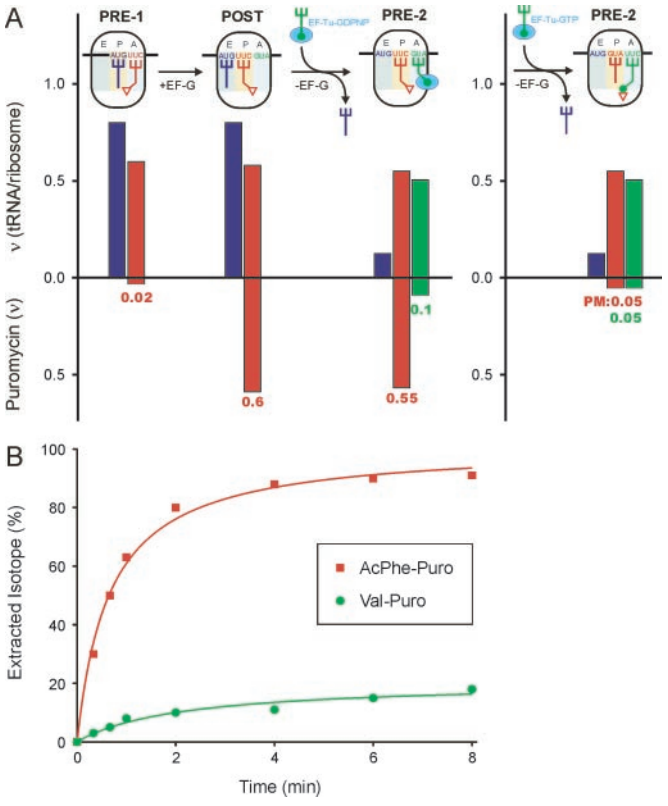

Figure 4. (**A**) tRNA binding and puromycin reaction with ribosomal complexes in different functional states. The binding of [^{32}P]tRNA$_f^{Met}$ (blue) to the P site and Ac[^{14}C]Phe-tRNA (red) to the A site (PRE-1 complex) was followed by addition of EF-G and GTP to induce translocation (POST). Next, the ternary complex EF-Tu•GDPNP•[^{3}H]Val-tRNA (green) was added and the new PRE-2 complex was formed. In all cases the extent of binding of the respective tRNAs are indicated with upright bars of the corresponding color, and the accessibility of peptidyl- and aa-tRNAs to puromycin is indicated with hanging bars of the same color. All data are expressed with ν, i.e. pmoles of bound tRNA or peptidyl- or aminoacyl-puromycin formed per pmole ribosome. The corresponding data from the PRE-2 state performed with GTP instead of GDPNP are taken from Figure 6A in (3). (**B**) Kinetics of the puromycin reaction of the PRE-2 complex. The complex carried Ac[^{14}C]Phe-tRNA at the P site and [^{3}H]Val-tRNA-EF-Tu-GDPNP at the A site. The puromycin concentration was 1 mM and the product was expressed according to both isotopes, either Ac[^{14}C]Phe-puromycin (squares) or [^{3}H]Val-puromycin (circles).

exact three steps was not addressed. Here we have tested whether or not E-tRNA can be released using a ternary complex [^{3}H]Val-tRNA•EF-Tu•GDPNP. Figure 4A (and Table 1 PRE-2) shows that the addition of this ternary complex with non-hydrolyzable GTP analogue GDPNP almost completely releases the E site. The A site was 60% occupied with [^{3}H]Val-tRNA•EF-Tu•GDPNP, and the occupancy of the tRNA$_f^{Met}$ dropped from 85% in the POST state to 15% in the PRE-2 state. Since the presence of GDPNP prevents

two subsequent reactions, viz. EF-Tu release and accommodation of the Val-tRNA at the A site, these results clearly demonstrate that the E-tRNA is released after the decoding process but before GTP hydrolysis, EF-Tu release and accommodation of an aa-tRNA at the A site. We reasoned that if this was really true, then the A site on the 50S should still be free for binding of puromycin since the CCA end of the A site tRNA is still bound to EF-Tu which is far from the PTC (15). Figure 4A shows that indeed the P site bound Ac[^{14}C]Phe-tRNA reacted almost quantitatively with puromycin, whereas the A site bound ternary complex containing Val-tRNA and GDPNP could not. The kinetics of the puromycin reaction shown in Figure 4B underline this observation. This behavior stands in striking contrast to the analogous experiment in the presence of GTP, where both Ac[^{14}C]Val and [^{3}H]Phe did not react with puromycin indicating dipeptide formation at the A site [Figure 4A, right panel, from Figure 6A in (3) for comparison].

CONCLUSIONS

The puromycin reaction is extremely sensitive owing to the low-affinity (K_M of ~0.2 mM) of this drug (16), such that an effect seen with the puromycin reaction might not occur when aa-tRNA is at the A site instead of puromycin (17). Here, we use this highly sensitive method of puromycin reactions and do not find a change of kinetic parameters of the reaction under any of various conditions. Therefore, it seems safe to conclude that peptide-bond formation does not in fact depend on the various functional states under observation.

The observation that a ternary complex triggers E-tRNA release after the decoding step but before GTP hydrolysis and accommodation of the aa-tRNA into the A site provides an elegant explanation of the finding that Pi and POST states show the same activity concerning peptide-bond formation. This observation means that the A-site region of the peptidyltransferase is occupied by the acylated end of the A-tRNA only after the E-tRNA release, i.e. peptide-bond formation always occurs in the presence of an empty E site, either when the E site is free *per se* (Pi state) or has become freed (POST state plus ternary complex).

We have found that a successful decoding event triggers E-tRNA release, but does not affect the active state of the PTC. An important implication of this finding is that the small subunit is a main player in the allosteric interactions between A and E site, supported by a wealth of data (3,5–7) without including the 50S region of the PTC, which is consistent with the large distance between the E site and PTC. In the future, it will be interesting to investigate how the E-tRNA is released in response to the binding of the tmRNA complexed with SmpB and EF-Tu•GTP (18,19), which binds to ribosomes stalled on truncated mRNAs that have no codon in the A site.

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Conflict of interest statement. None declared.

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