Rapid peptide bond formation on isolated 50S ribosomal subunits

Ingo Wohlgemuth, Malte Beringer & Marina V. Rodnina⁺

Institute of Physical Biochemistry, University of Witten/Herdecke, Witten, Germany

The catalytic site of the ribosome, the peptidyl transferase centre, is located on the large (50S in bacteria) ribosomal subunit. On the basis of results obtained with small substrate analogues, isolated 50S subunits seem to be less active in peptide bond formation than 70S ribosomes by several orders of magnitude, suggesting that the reaction mechanisms on 50S subunits and 70S ribosomes may be different. Here we show that with full-size fMet-tRNA^{fMet} and puromycin or C-puromycin as peptide donor and acceptor substrates, respectively, the reaction proceeds as rapidly on 50S subunits as on 70S ribosomes, indicating that the intrinsic activity of 50S subunits is not different from that of 70S ribosomes. The faster reaction on 50S subunits with fMet-tRNA^{fMet}, compared with oligonucleotide substrate analogues, suggests that full-size transfer RNA in the P site is important for maintaining the active conformation of the peptidyl transferase centre.

Keywords: ribozymes; translation; rapid kinetics; ribosomes

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INTRODUCTION

Polypeptides are synthesized by the ribosome, a large particle that is made up of ribosomal RNAs (rRNA) and proteins. The bacterial 70S ribosome consists of two subunits, small (30S) and large (50S). The active site for peptide bond formation (peptidyl transferase centre) is located on the 50S subunit (Nissen *et al*, 2000; Bashan *et al*, 2003; Schmeing *et al*, 2005a,b). The substrates of the reaction, peptidyl-transfer RNA (tRNA) and aminoacyl-tRNA (aa-tRNA), bind to the P and A sites of the ribosome, respectively. The reactions catalysed by the 70S ribosome and the 50S subunit are similar in a number of ways: they use the same substrates, they are susceptible to the same inhibitors (Krayevsky & Kukhanova, 1979; Moore & Steitz, 2003) and they have similar pH-rate profiles (Maden & Monroe, 1968; Katunin *et al*, 2002; Okuda *et al*, 2005). So far, the reaction on the 50S subunit has been studied mainly using short substrate analogues lacking the 30S subunit-binding part of the tRNA molecule as peptide donor substrates

(fragment reaction; Monroe, 1967; Krayevsky & Kukhanova, 1979; Sardesai *et al*, 1999; Schmeing *et al*, 2002; Okuda *et al*, 2005; Seila *et al*, 2005). In these assays, the rate of product formation was low, usually orders of magnitude slower than on the 70S ribosome (Moore & Steitz, 2003). The large difference in rate could imply that the structure of the peptidyl transferase centre in the 70S ribosome deviates significantly from that in the isolated 50S subunit, or even that there is a mechanistic difference between the reactions on the 70S ribosome and the isolated 50S subunit (Moore & Steitz, 2003).

RESULTS

We addressed the question of the intrinsic catalytic proficiency of the 50S subunit by comparing 50S subunits and 70S ribosomes with respect to the rates of peptide bond formation between the natural P-site substrate fMet-tRNA^{fMet} and analogues of aa-tRNA, puromycin (Pmn) or puromycin with an additional cytidine residue equivalent to C75 of tRNA (CPmn), as A-site substrates. Initially, the data obtained with 50S subunits showed large variability. This could be eliminated by removing traces of contaminating 30S subunits from the preparations of 50S subunits. An efficient purification was achieved by incubating preparations of 50S subunits with biotinylated deoxyoligonucleotides, which anneal at regions of 16S rRNA that are readily accessible in the 30S subunit (Fuchs *et al*, 1998), followed by removal of the oligonucleotide-tagged 30S subunits from the mixture using streptavidin-coated magnetic beads (Fig 1).

For peptide bond formation to occur the 50S subunit has to bind its two substrates, fMet-tRNA^{fMet} and Pmn (or CPmn), to the P and A sites, respectively (Fig 2A). The reaction products, tRNA^{fMet} in the P site and fMet-Pmn (fMet-CPmn) in the A site, are readily exchanged for new substrates, which are present in excess, leading to multiple rounds of the reaction. To estimate the turnover rate constant, k_{cat} , the rate of peptide bond formation was determined as a function of the concentrations of both substrates at steady-state initial-velocity conditions. The k_{cat} value represents the lower limit of the rate constant of the chemistry step and k_{pep} represents other steps of the reaction (structural rearrangements, product release) that might influence the turnover rate. The affinity of Pmn to the 70S ribosome (Katunin *et al*, 2002; Brunelle *et al*, 2006) or the 50S subunit (Fig 2B) is 3–4 mM (37 °C). The apparent affinity of CPmn to the 50S subunit, 0.2 mM, is higher than that to

Institute of Physical Biochemistry, University of Witten/Herdecke, Stockumer Strasse 10, 58448 Witten, Germany

⁺Corresponding author. Tel: +49 2302 926205; Fax: +49 2302 926117;

E-mail: rodnina@uni-wh.de

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Fig 1 | Purification of 50S subunits. (A) Rate of fMet-Pmn formation on 50S subunits (20 mM Mg²⁺). (1, 2, 3) Reactions were carried out with unpurified 50S subunits containing 0.6% (1, 2) or 2.8% (3) 70S ribosomes; (2) 50S subunits preincubated for 1 h at 37 °C. (4, 5, 6) Same as (1, 2, 3), respectively, with purified 50S subunits. 50S subunits (0.6 μM) were incubated with fMet-tRNA^{fMet} (6.6 μM) and Pmn (10 mM) for 5 min at 37 °C. Standard deviation of measured values was <10%. (B) Dot-blot analysis. Upper panel, different amounts of 50S subunits containing 0.6% 30S subunits visualized using an antibody against ribosomal protein S7. Lower panel, 50S subunits (40 pmol) purified by treatment with biotinylated DNA-oligonucleotides Eco1410 or Eco1482 complementary to nucleotides 1,410–1,427 and 1,482–1,499 of 16S ribosomal RNA, respectively, at different hybridization temperatures. After treatment with Eco1410 at 50 °C, >90% of the contamination was removed, as quantified by densitometry. (C) fMet-Pmn formation on 70S initiation complexes. Initiation complexes (IC) were prepared from purified (filled circle) and non-purified (open circle) 50S subunits. Time courses were measured by quench-flow with initiation complexes (0.5 μM) and Pmn (1 mM; 7 mM Mg²⁺, 37 °C). The rate of fMet-Pmn formation was about 0.5 s⁻¹ in both cases. Pmn, puromycin.

the 70S ribosome, 1.7 mM (Brunelle *et al*, 2006). Binding of fMet-tRNA^{fMet} to the P site of the 50S subunit is weak, owing to the lack of tRNA interactions with the 30S subunit. The low affinity is

Fig 2 | Peptide bond formation on 50S subunits. (A) Reaction scheme. Step 1, binding of the substrates, fMet-tRNA^{fMet} and Pmn, to P and A sites, respectively; step 2, peptidyl transfer; step 3, dissociation of products, deacylated transfer RNA and fMet-Pmn. (B) Rate dependence on Pmn (filled square; left y axis) or CPmn (filled triangle; right y axis) concentration. The reaction was carried out by mixing 50S subunits (0.6 μM) with fMet-tRNA^{fMet} (6.6 μM) and increasing concentrations of Pmn (CPmn) (100 mM Mg²⁺). (C) Mg²⁺ dependence of initial velocity. Initial velocities were measured with 50S subunits (0.6 μM), fMet-tRNA^{fMet} (6.6 μM, non-saturating concentration) and Pmn (10 mM, saturating concentration). (D) fMet-tRNA^{fMet} titration. The reaction was performed with 50S subunits (0.6 μM), Pmn (10 mM) (filled square; left y axis) or CPmn (0.6 mM) (filled triangle; right y axis), and increasing fMet-tRNA^{fMet} concentrations at 100 mM Mg²⁺. Solid lines represent satisfactory fits to a Michaelis-Menten equation (see text). Dashed lines represent fits that deviate significantly from the experimental data, which give the lower limit for the extrapolated k_{cat} value. Pmn, puromycin.

one possible reason for the previously observed low rate of reaction on the 50S subunit, as substrates were used at concentrations far below saturation. The affinity of tRNA binding

to the ribosome is increased at elevated Mg^{2+} concentrations. In fact, the velocity of the reaction measured at limiting concentrations of fMet-tRNA^{fMet} and saturating Pmn increased with Mg^{2+} concentration up to about 100 mM (Fig 2C). Furthermore, the reaction rate increased with the concentration of fMet-tRNA^{fMet} and reached a velocity of 0.008 s^{-1} with Pmn or 0.4 s^{-1} with CPmn at the highest concentration of fMet-tRNA^{fMet} that was attainable at about 50–60 μM (Fig 2D). The linear increase of the reaction velocity with concentration indicates that only the initial part of the Michaelis–Menten curve was covered, and the maximum rate must be much higher. For reaction with Pmn, modelling the Michaelis–Menten curve using $k_{\text{cat}} = 0.17 \text{ s}^{-1}$, the rate constant of the reaction on the 70S ribosomes (see below), and $K_{\text{M}} = 1.1 \text{ mM}$, calculated from $k_{\text{cat}}/K_{\text{M}} = 1.6 \times 10^{-4} \mu\text{M}^{-1} \text{ s}^{-1}$ (slope in Fig 2D), gave a satisfactory fit to the data (solid line in Fig 2D). In contrast, a clear deviation from the experimental data was obtained when $k_{\text{cat}} = 0.05 \text{ s}^{-1}$ and $K_{\text{M}} = 313 \mu\text{M}$ were assumed (Fig 2D, dashed line). For reaction with CPmn, the best fit (solid line in Fig 2D) was obtained assuming $k_{\text{cat}} = 19 \text{ s}^{-1}$, the rate constant of fMet-CPmn formation on 70S ribosomes (data not shown; Brunelle *et al*, 2006), and $K_{\text{M}} = 3.2 \text{ mM}$, calculated from the value $k_{\text{cat}}/K_{\text{M}} = 6 \times 10^{-3} \mu\text{M}^{-1} \text{ s}^{-1}$. A clear deviation from the data was observed when $k_{\text{cat}} = 1 \text{ s}^{-1}$ and $K_{\text{M}} = 0.17 \text{ mM}$ were assumed (dashed line). This indicates that the rate constant of peptide bond formation on the 50S subunit must be approximately 0.17 s^{-1} with Pmn and approximately 10 s^{-1} with CPmn.

When measurements with 70S ribosomes were performed under steady-state conditions, that is, when both substrates (fMet-tRNA^{fMet} and Pmn) were added simultaneously, a rapid burst phase was observed that yielded $k_{\text{pep}} = 0.5 \text{ s}^{-1}$ at 20 mM Mg^{2+} and was followed by a slow turnover (Fig 3A). Alternatively, k_{pep} was measured at single-turnover conditions with the same substrates, yielding a similar k_{pep} value of 0.33 s^{-1} (Fig 3B, circles). The rate of peptide bond formation on 70S ribosomes was only slightly influenced by the Mg^{2+} concentration, decreasing from 0.6 s^{-1} at 7 mM Mg^{2+} (Katunin *et al*, 2002) to 0.17 s^{-1} at 100 mM Mg^{2+} (Fig 3B). This suggests that catalysis is not affected by Mg^{2+} , indicating that the enhancement of the activity of the 50S subunits with increasing Mg^{2+} concentration was owing to enhanced tRNA binding.

DISCUSSION

The present data indicate that the catalytic activity of the isolated 50S subunit is very similar to that of the 70S ribosome when full-size fMet-tRNA^{fMet} is used as the P-site substrate. This finding lends support to mechanisms of the peptidyl transfer reaction derived from crystal structures of isolated 50S subunits. Most importantly, the present results argue against models that imply essential conformational changes of the peptidyl transferase centre on the 50S subunit, which are induced by binding of the 30S subunit.

Significantly lower rates of peptide bond formation on isolated 50S subunit were previously reported, and there are several likely explanations for this difference. First, in the early work, velocities were not measured at substrate saturation (Monro, 1967; Krayevsky & Kukhanova, 1979). Second, 3'-terminal tRNA fragments rather than full-size tRNA were used. In fact, the reaction on the 50S subunit was faster when full-size Ala-tRNA^{Ala}, rather than a short fragment of the same tRNA, was used as a P-site

Fig 3 | Peptide bond formation on 70S ribosomes. (A) Steady-state conditions. The reaction was initiated by rapidly mixing 70S ribosomes (0.36 μM , 10 pmol), Pmn (10 mM) and fMet-tRNA^{fMet} (6.6 μM) at 20 mM Mg^{2+} . The rate of the exponential burst phase was $0.5 \pm 0.1 \text{ s}^{-1}$, the burst intercept was 9 pmol and the rate of the linear turnover phase was 0.1 s^{-1} . (B) Pre-steady-state conditions. Time courses were measured with 70S initiation complexes (0.25 μM) and Pmn (1 mM) at Mg^{2+} concentrations of 7 mM (filled square, $k_{\text{pep}} = 0.52 \pm 0.01 \text{ s}^{-1}$), 20 mM (filled circle, $k_{\text{pep}} = 0.33 \pm 0.02 \text{ s}^{-1}$) and 100 mM (filled triangle, $k_{\text{pep}} = 0.17 \pm 0.01 \text{ s}^{-1}$). Pmn, puromycin.

substrate (Sardesai *et al*, 1999). This raises the possibility that an interaction by part of the tRNA molecule beyond the 3'-terminal sequence induces a conformational change of the peptidyl transferase centre, thus enhancing the catalytic activity. Structural studies indicate that the binding of aa-tRNA analogues in the A site induced specific movements in 23S rRNA, thereby re-orientating the ester group of the peptidyl-tRNA and making it accessible for the attack by the A-site nucleophile (Schmeing *et al*, 2005b). The present data indicate that the adjustment of the P-site substrate has an effect on catalysis as well. Thus, the presence of both A- and P-site tRNAs seem to be important for inducing, or maintaining, the active conformation of the peptidyl transferase centre by induced fit.

Except for the contacts in the peptidyl transferase centre itself, tRNA interactions with the 50S subunit involve elements (helix 69, protein L5) that contact the elbow region of the P-site tRNA (Yusupov *et al*, 2001). These contacts may form with fMet-tRNA^{fMet}, but not with oligonucleotide substrate analogues. The contact site on the tRNA may be further narrowed down to the T stem-loop and the acceptor stem of tRNA, because Ala-tRNA^{Ala} and an Ala-tRNA minihelix were equally active on 50S subunits, whereas a 9-mer 3'-terminal fragment of the same tRNA had a much lower activity (Sardesai *et al*, 1999). The only contact of the

T stem with the 50S subunit is through protein L5, which may, therefore, be involved in modulating the peptidyl transferase activity.

As the activity of 50S subunits and the 70S ribosomes are equal, the data obtained for 50S subunits (crystal structures, molecular dynamics simulations) and for 70S ribosomes (kinetics, biochemistry, mutagenesis) may be taken together to support the current model of the mechanism of peptide bond formation. In summary, the ribosome enhances the rate of peptide bond formation by reducing the activation entropy (Sievers *et al*, 2004), which might be due to substrate positioning and exclusion of water from the active site (Sievers *et al*, 2004; Sharma *et al*, 2005; Trobro & Åqvist, 2005). The ribosome seems to provide a preorganized electrostatic environment that stabilizes the polar transition state (Schmeing *et al*, 2005a; Trobro & Åqvist, 2005). The reaction does not involve chemical catalysis by ribosomal groups, but might be modulated by conformational changes at the active site (Polacek *et al*, 2001; Thompson *et al*, 2001; Beringer *et al*, 2003, 2005; Youngman *et al*, 2004; Bieling *et al*, 2006; Brunelle *et al*, 2006). The 2'-OH of A76 of the P-site tRNA has a crucial role in the reaction, both on isolated 50S subunits (Krayevsky & Kukhanova, 1979) and 70S ribosomes (Weinger *et al*, 2004), but not in the uncatalysed reaction (Sharma *et al*, 2005). The most favourable mechanism of catalysis involves an intrareactant proton shuttling through the 2'-OH of A76 of the P-site tRNA, which follows the attack of the A-site α -amino group on the P-site ester (Schmeing *et al*, 2005a; Trobro & Åqvist, 2005). Interactions of the tRNA body with the ribosome might be required to align precisely the tRNA relative to the 50S subunit and thus optimize and stabilize the orientation of the groups involved in the proton shuttle. Finally, the peptidyl transferase centre seems to be activated by conformational changes induced by substrate binding not only to the A site (Schmeing *et al*, 2005b; Brunelle *et al*, 2006) but also to the P site.

METHODS

Buffers and reagents. Experiments were carried out in buffer A (50 mM Tris-HCl, 20 mM Bis-Tris-HCl pH 7.5, 70 mM NH₄Cl, 30 mM KCl and MgCl₂ as indicated) at 37 °C. 70S ribosomes and ribosomal subunits from MRE600, initiation factors and [³H]Met-tRNA^{fMet} were prepared as described (Rodnina & Wintermeyer, 1995; Rodnina *et al*, 1999). MF-mRNA (5'-GGCAAGGAGGU AAAUAAUGUUCACGAUU-3', initiation codon underlined) was purchased from Dharmacon Research Inc. (Boulder, CO, USA). Radioactive L-methionine was purchased from MPBiomedicals (Eschwege, Germany) and streptavidin-coated magnetic beads were purchased from Roche Diagnostics (Mannheim, Germany). All other chemicals were obtained from Sigma (Steinheim, Germany) or Merck (Darmstadt, Germany).

Purification of 50S subunits. To remove trace quantities of 30S subunits in 50S subunit preparations, 3'-biotinylated DNA-oligonucleotides Eco1410 (GCAACCCACTCCCATGGT) or Eco1482 (TACGACTTCACCCCAGTC) (0.5 μ M; Fuchs *et al*, 1998; Bio TEG, Operon, Huntsville, AL, USA) complementary to nucleotides 1,410–1,427 and 1,482–1,499 of 16S rRNA, respectively, were annealed to 30S subunits present in the 50S subunit preparations (5 μ M) in a typical volume of 150 μ l. The Mg²⁺ concentration in the reaction was adjusted to 2 mM to favour subunit dissociation. After incubation, the mixture was added to streptavidin-coated magnetic beads and incubated at 20 °C for

30 min. The beads were pulled out of solution with a magnet, and the supernatant containing purified 50S subunits was recovered.

Immunodetection of 30S subunits was carried out using a polyclonal antibody against ribosomal protein S7. Detection was performed by chemoluminescence. X-ray films were exposed for 30 s, and spots were quantified densitometrically.

Steady-state puromycin reaction. If not stated otherwise, purified 50S subunits (0.66 μ M) were incubated with Pmn or CPmn and [³H]Met-tRNA^{fMet} in 15 μ l of buffer A at 37 °C. The stock solution of Pmn (106 mM; Sigma, Steinheim, Germany) contained 20% dimethyl sulphoxide (DMSO) and its pH was adjusted to 7.5 by 5 M KOH. The presence of up to 20% DMSO had no effect on the rate of the reaction (data not shown). Where indicated, 50S subunits were preincubated in buffer A with 20 mM MgCl₂ for 1 h at 37 °C (Noll *et al*, 1973). Reactions were quenched with 0.5 M KOH, incubated for 45 min at 37 °C to hydrolyse unreacted [³H]Met-tRNA^{fMet} and neutralized with acetic acid. [³H]Met-Pmn and [³H]Met were separated on an RP-8 column and quantified by radioactivity counting. In the absence of 50S subunits, no product was found after 5 min incubation at all conditions. The rates were calculated from up to eight independent experiments.

Peptide bond formation on 70S ribosomes. 70S ribosomes were prepared by incubating purified 50S subunits with stoichiometric amounts of 30S subunits for 1 h at 37 °C in buffer A containing 20 mM MgCl₂. Initiation complexes were prepared in buffer A by incubating 70S ribosomes (0.4 μ M), MFT-mRNA (0.68 μ M), [³H]Met-tRNA^{fMet} (0.68 μ M), initiation factors 1, 2 and 3 (0.68 μ M each) and GTP (1 mM) for 60 min at 37 °C. The initiation efficiency (>95%) was controlled by nitrocellulose filtration. Filters were dissolved in 10 ml of scintillation liquid (Quickszint 361; Zinsser Analytic, Frankfurt, Germany) and counted in a TriCarb counter (Perkin-Elmer, Boston, MA, USA).

Time courses of [³H]Met-Pmn formation on 70S initiation complexes on addition of Pmn (1 mM) and time courses on vacant 70S ribosomes on addition of [³H]Met-tRNA^{fMet} (6.6 μ M) and Pmn (10 mM) were measured in buffer A at 37 °C by mixing equal volumes (14 μ l) of ribosomes (0.8 μ M after mixing) or initiation complexes (0.25 μ M) and Pmn in a quench-flow apparatus (KinTek Corp., Austin, TX, USA). Time courses of single-round measurements were evaluated by single-exponential fitting; steady-state burst measurements on 70S ribosomes were evaluated by fitting to a sum of an exponential and a linear term.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>)

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