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Important Contribution to Catalysis of Peptide Bond Formation by a Single Ionizing Group within the Ribosome

Vladimir I. Katunin,[1] Gregory W. Muth,[2] Scott A. Strobel,[2] Wolfgang Wintermeyer,[3] and Marina V. Rodnina[4,5]

[1]Sankt-Petersburg Nuclear Physics Institute
Russian Academy of Sciences
Gatchina 188350
Russia

[2]Department of Molecular Biophysics and Biochemistry
Yale University
New Haven, Connecticut 06520

[3]Institute of Molecular Biology

[4]Institute of Physical Biochemistry
University of Witten/Herdecke
58448 Witten
Germany

Summary

The catalytic mechanism of peptide bond formation on the ribosome is not known. The crystal structure of 50S ribosomal subunits shows that the catalytic center consists of RNA only and suggests potential catalytic residues. Here we report rapid kinetics of the peptidyl transferase reaction with puromycin at rates up to 50 s^{-1}. The rate-pH profile of the reaction reveals that protonation of a single ribosomal residue (pK_a = 7.5), in addition to protonation of the nucleophilic amino group, strongly inhibits the reaction (>100-fold). The A2451U mutation within the peptidyl transferase center has about the same inhibitory effect. These results suggest a contribution to overall catalysis of general acid-base and/or conformational catalysis involving an ionizing group at the active site.

Introduction

The elongation cycle of protein synthesis is comprised of three major steps. During the first step, aminoacyl-tRNA (aa-tRNA) with an anticodon complementary to the mRNA codon presented in the decoding site binds to the A site of the ribosome. The decoding site is located on the small ribosomal subunit called 30S in bacterial ribosomes. In the second step, A site-bound aa-tRNA reacts with P site-bound peptidyl-tRNA (pept-tRNA) to form a peptide bond, resulting in deacylated tRNA in the P site and pept-tRNA that is one amino acid longer in the A site. The reaction takes place in the peptidyl transferase (PT) center that is located on the large ribosomal subunit 50S in bacteria. The cycle is completed by the translocation of pept-tRNA from the A site to the P site; during the movement, the mRNA is carried along with the tRNA, performing a movement by one codon triplet, and deacylated tRNA moves out of the P site and dissociates from the ribosome. In order to proceed at physiologically relevant rates, the first and the third steps require catalysis by accessory enzymes, elongation factors (EF). In the bacterial system, these factors are EF-Tu, which delivers aa-tRNA to the ribosome, and EF-G, which promotes translocation. The second step, peptide bond formation, is catalyzed by the ribosome and does not involve accessory factors. The reaction proceeds via nucleophilic attack of the α-amino group of aa-tRNA on the carbonyl ester bond of pept-tRNA. This leads to a tetrahedral intermediate that is subsequently resolved into a deacylated tRNA (P site) and a new, one amino acid longer, pept-tRNA (A site). The catalytic mechanism of the reaction on the ribosome and the ribosomal groups involved in catalysis are not known.

50S subunits largely depleted of protein retained substantial PT activity, suggesting that the activity might reside in 23S rRNA (Noller et al., 1992), although a contribution by protein could not be completely excluded (Diedrich et al., 2000; Garrett and Rodriguez-Fonseca, 1996; Khaitovich et al., 1999). The atomic structure of the large ribosomal subunit from the archaeon *Haloarcula marismortui* shows that the PT center is composed of RNA exclusively (Ban et al., 2000; Nissen et al., 2000), and a similar structure was observed in 50S subunits from *Deinococcus radiodurans* (Harms et al., 2001). Positioning of the 3′ CCA ends of both pept-tRNA and aa-tRNA by forming base pairs with rRNA appears to have an important role in catalysis (Kim and Green, 1999; Samaha et al., 1995). Several base pairs between CCA sequences of substrate analogs and bases in the P and A loops of the PT center were revealed by the 50S crystal structures (Nissen et al., 2000).

Additional contributions to catalysis, such as general acid-base catalysis, have been discussed previously (Garrett and Rodriguez-Fonseca, 1996; Krayevsky and Kukhanova, 1979; Lieberman and Dahlberg, 1995; Nierhaus et al., 1980). Early studies of the PT reaction, using peptidyl- or polyphenylalanyl-tRNA as donor substrate in the P site and puromycin (Pm) as A site substrate, revealed a strong pH dependence of the reaction. This suggested a contribution of general acid-base catalysis involving an ionizing group with a pK_a of 7.5–8.0 (Maden and Monro, 1968) or 7.2 (Pestka, 1972). A similar pH dependence was observed using a Pm analog with a hydroxyl group replacing the α-amino group (Fahnestock et al., 1970), indicating that the reaction was in fact strongly impaired by protonation of a functional group of the ribosome. However, in these early studies the measured rates were generally rather low (<2 min^{-1}), which suggests that the observed reaction rates were limited by some step(s) other than the chemical step. On the basis of the crystal structure, a conserved adenine residue (A2486 in *H. marismortui*, A2451 in *Escherichia coli*; *E. coli* numbering is used hereafter) in the PT center was suggested to act as a general base (Nissen et al., 2000). Mutational analysis in vivo implied an essential role of A2451 for the function of *E. coli* ribosomes (Muth et al., 2000). However, kinetic data on the catalytic step necessary to substantiate such models are lacking so far.

In the present work, the kinetics of the PT reaction were studied under conditions where the catalytic step

[5]Correspondence: rodnina@uni-wh.de

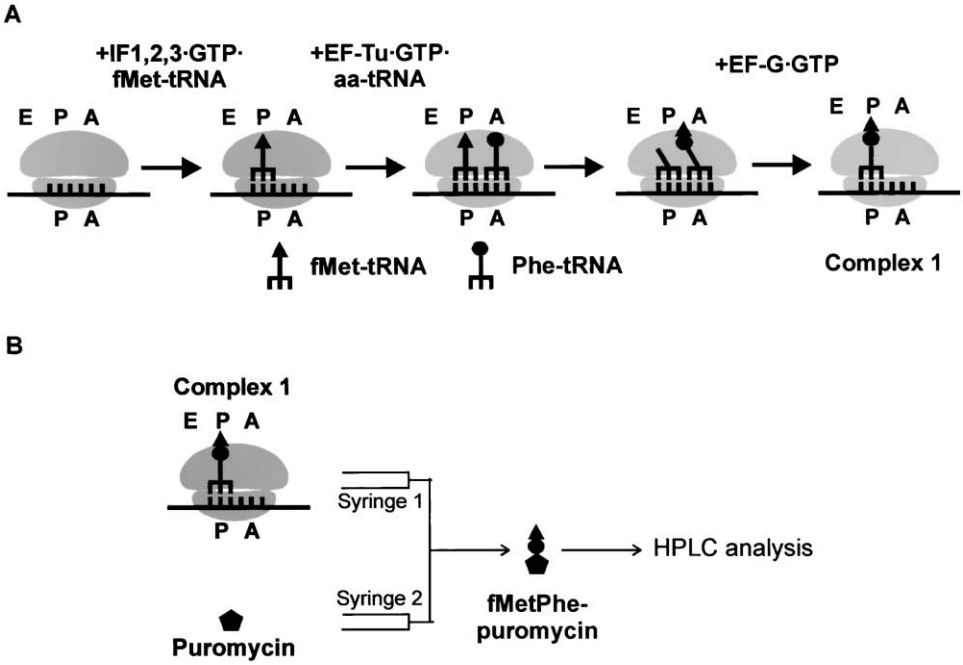

Figure 1. Preparation of Ribosome Complexes and Rapid Kinetic Assay of PT Activity

of the PT reaction was rate limiting and rates up to 50 s^{-1} were attained. Rapid kinetic experiments were performed by the quench-flow technique, monitoring the PT reaction with puromycin (Pm) on fully active ribosomes carrying pept-tRNA in the P site. The quench-flow assay was used to study the pH dependence of the PT reaction and to assess the influence of the A2451U substitution in the PT center on the rate of the reaction.

Results

Rapid Kinetics of Peptide Bond Formation

Pre-steady state kinetic measurements have shown that peptide bond formation between pept-tRNA in the P site and aa-tRNA in the A site is rapid and rate limited by the preceding step of aa-tRNA accommodation in the PT center (rate constant 7 s^{-1}, 20°C) (Pape et al., 1998). In order to circumvent the accommodation step and directly measure the rate of the catalytic step, we have developed a quench-flow assay that uses Pm as A site substrate and pept-tRNA, f[^{3}H]Met[^{14}C]Phe-tRNA^{Phe}, synthesized on the ribosome, as P site substrate. This assay was then used to measure the pH dependence of the PT reaction.

Pept-tRNA was synthesized on *E. coli* ribosomes programmed with natural mRNA by performing initiation and one complete elongation cycle (Figure 1). The 70S initiation complex was prepared by binding f[^{3}H]Met-tRNA^{fMet} to the P site in the presence of initiation factors. Subsequently, [^{14}C]Phe-tRNA^{Phe} was bound to the A site from the ternary complex with EF-Tu·GTP. Peptide bond formation resulted in f[^{3}H]Met[^{14}C]Phe-tRNA^{Phe} in the A site, which was translocated to the P site upon addition of EF-G·GTP (Figure 1A). The stable postranslocation complex was purified from translation factors and unbound fMet- and Phe-tRNA by ultracentrifugation (complex 1) and was used for the quench-flow experiments (Figure 1B). All steps proceeded to completion, resulting in >90% homogeneous complex 1. After reacting complex 1 with excess Pm in a quench-flow apparatus, the reaction was quenched by KOH. The f[^{3}H]Met[^{14}C]Phe-Pm formed by the PT reaction and unreacted f[^{3}H]Met[^{14}C]Phe set free from f[^{3}H]Met[^{14}C]Phe-tRNA^{Phe} by alkaline hydrolysis were separated by HPLC and quantitated by double-label radioactivity counting (Rodnina and Wintermeyer, 1995). This way the product measured by quantitative analysis was characterized as f[^{3}H]Met[^{14}C]Phe-Pm formed on fully active ribosomes that went through initiation and one complete elongation cycle.

Traditional assays used Pm at 0.1–1 mM concentration (Maden and Monro, 1968; Pestka, 1972). At 1 mM Pm, fMetPhe-Pm was formed at a rate of 2 s^{-1} in the present assay system (pH 7.2; 37°C). The rate of the reaction increased with Pm concentration and reached a plateau at 10 mM at both pH values (7.2 and 5.7) tested (Figure 2). This behavior suggested that at high Pm concentration the rate of the bimolecular binding step was no longer rate limiting and that the observed rate represented the rate constant of the catalytic step, k_{pep}. At pH 7.2, k_{pep} was about 18 s^{-1}, i.e., in the range compatible with in vivo rates of translation. At pH 5.7, k_{pep} was 300 times smaller. The concentration dependence was the same, indicating that the K_d of Pm binding to the ribosome, about 3 mM, was independent of pH.

The possibility remains that a conformational change that affected Pm binding to the A site was rate limiting in the assay. To explore this possibility, we measured rates of the Pm reaction with various pept-tRNA substrates in the P site, reasoning that these rates should be the same if some step of A site binding was rate limiting. Rate constants of peptide bond formation mea-

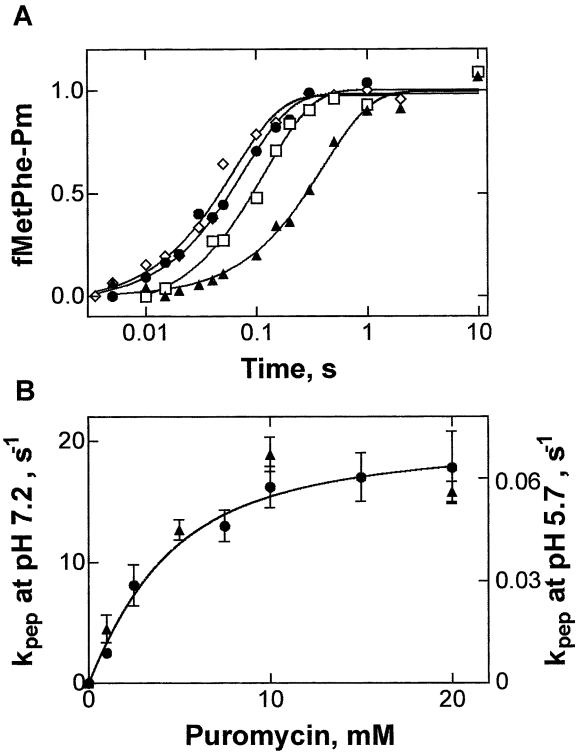

Figure 2. Dependence of PT Kinetics on Pm Concentration

(A) Time curves of fMetPhe-Pm formation. Quench-flow assays were performed using complex 1 (Figure 1) in buffer A at 37°C at Pm concentrations of 1 (closed triangles), 2.5 (open squares), 10 (closed circles), and 20 (open diamonds) mM. From the time curves, apparent rate constants (k_{pep}) were obtained by single-exponential fitting (continuous lines).

(B) Dependence of k_{pep} on Pm concentration. k_{pep} values obtained at pH 7.2 (circles) and pH 5.7 (triangles) are plotted against the concentration of Pm; standard deviations are indicated by error bars. Fitting the data to a two-step model where a reversible binding step is followed by an irreversible reaction yielded $k_{pep} = 18\ s^{-1}$ (pH 7.2) and $0.06\ s^{-1}$ (pH 5.7) at saturation and an apparent K_d of 3 mM at both conditions.

sured at saturating Pm concentration (10 mM) were rather different, ranging from $0.8\ s^{-1}$ for fMet-tRNA^{fMet} to $45\ s^{-1}$ for fMetAlaAsnMetPheAla-tRNA^{Ala} (Figure 3). This shows that the reaction is not limited by any step related to Pm binding into the reactive position in the A site and suggests that the quench-flow assay measures a step directly belonging to the catalysis of peptide bond formation. That the assay monitors the chemistry step is also suggested by results obtained with a derivative of puromycin in which the amino group was exchanged for a hydroxyl group (see below).

Two Ionizing Groups in the Peptidyl Transferase Reaction with Pm

The pH dependence of the PT reaction between fMet-Phe-tRNA^{Phe} and Pm was determined from time curves measured at saturating Pm concentration and at pH values between pH 5.3 and 8.7, yielding rate constants of the PT reaction, k_{pep}. The ribosome complexes remained intact and competent in peptide bond formation over the whole pH range. This was shown by unchanged

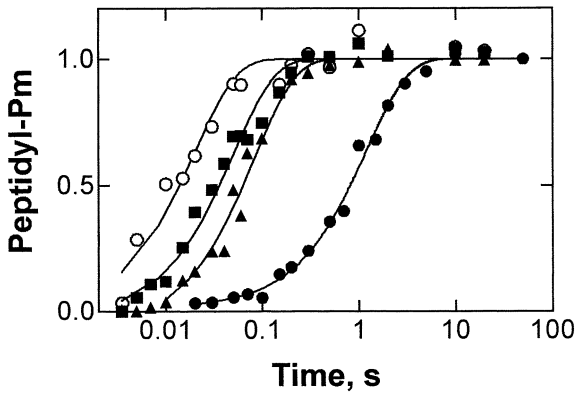

Figure 3. Dependence of PT Kinetics on P Site Substrate

Ribosomal complexes carrying different pept-tRNAs in the P site. f[^{3}H]Met-tRNA^{fMet} (closed circles), f[^{3}H]Met[^{14}C]Phe-tRNA^{Phe} (triangles), f[^{3}H]Met[^{14}C]Phe[^{14}C]Phe-tRNA^{Phe} (squares), f[^{3}H]Met[^{14}C]Ala-AsnMetPhe[^{14}C]Ala-tRNA^{Ala} (open circles). Quench-flow assays were performed in buffer A (pH 7.2; 37°C) with 10 mM Pm as in Figure 1.

levels of fMetPhe-tRNA^{Phe} binding and reactivity with Pm (close to 100%, i.e., one per ribosome) at all pH values tested (Figure 4A). All time curves followed single-exponential kinetics and were highly reproducible in independent experiments. The plot of k_{pep} versus pH showed a strong pH dependence in which k_{pep} reached a plateau of about $50\ s^{-1}$ at pH values >7.8 (Figure 4A). The smallest value of k_{pep}, $0.05\ s^{-1}$, was measured at pH 5.3; lower pH values were not accessible experimentally. Variations of buffer concentration had no effect on the observed reaction rates (data not shown).

The pH dependence of the reaction provides important insights into the nature of the reaction pathway. The maximum value of k_{pep}, $50 \pm 10\ s^{-1}$, and the highest pK_a value of the ionizing group(s) being titrated, $pK_a = 7.5 \pm 0.1$, were determined from the plot of $\log(k_{pep})$ versus pH (Figure 4B). This pK_a is significantly higher than the pK_a of Pm, which was determined to be 6.9 ± 0.2 (37°C) (Figure 4C) (see also Smith et al., 1965).The slope of the plot was 1.5 (Figure 4B). It should be 1.0 if there was only one ionizing group involved (cf. modeled curve in Figure 4B). This argues that at least two ionizing groups take part in the PT reaction with Pm. The fact that the slope was 1.5, and not 2.0, suggested that the singly protonated intermediate can still react at a significant rate.

The unknown rate constant of the reaction in the singly protonated system and the unknown pK_a of the second base were estimated from the $\log(k_{pep})$ versus pH plot (Figure 4B). The system was modeled as having two ionizing groups (Fersht, 1998) , one with pK_{a1} of the substrate, Pm, and another with pK_{a2} of a ribosomal residue, Rs, and assuming that protonation of the nucleophilic substrate virtually eliminates the reaction. For the calculations, the following parameters were used: $k_{pep} < 0.01\ s^{-1}$ for the doubly protonated system, $k_{pep} = 50\ s^{-1}$ for the unprotonated system, and $pK_{a2} = 7.5$. The best fit (Figure 4B) was obtained with the parameters summarized in Figure 4D. This suggests that (1) single protonation ($pK_{a2} = 7.5$) reduces the rate constant of the PT reaction about 100-fold, to $0.5 \pm 0.2\ s^{-1}$, and (2)

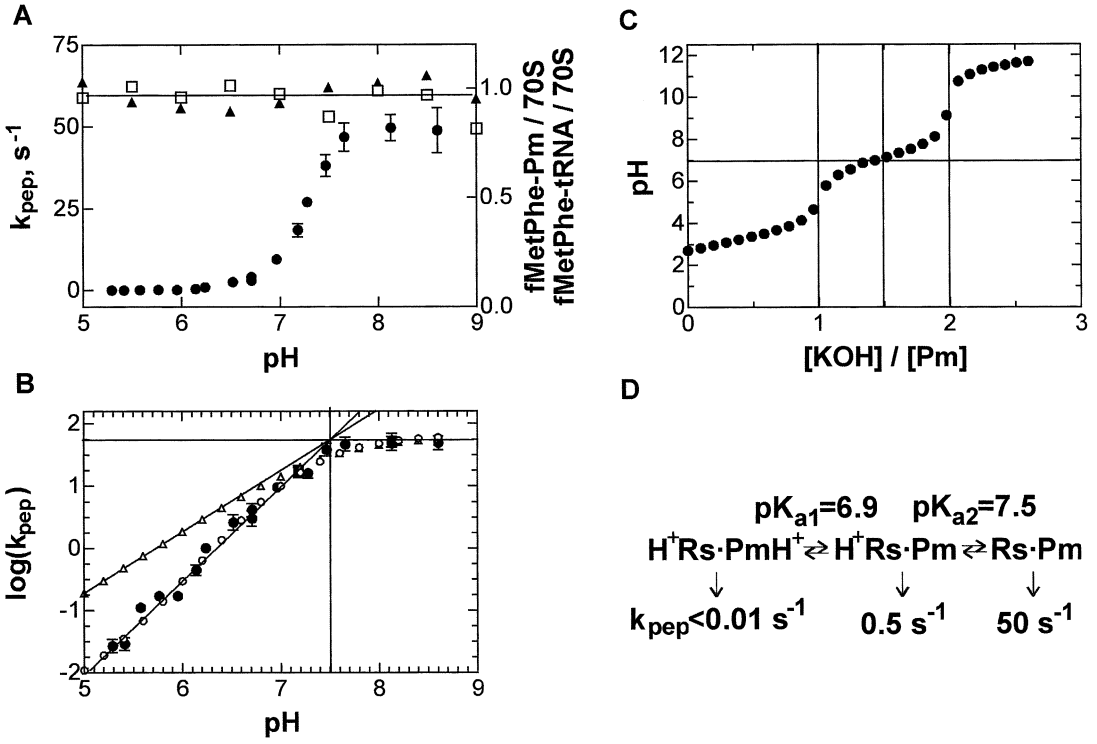

Figure 4. pH Dependence of fMetPhe-Pm Formation

(A) pH dependence of k_{pep}. Quench-flow assays were performed with complex 1 (Figure 1) and 10 mM Pm at 37°C. k_{pep} values (circles) were obtained by single-exponential fitting of time curves (cf. Figure 2). In parallel, the amount of f[^{3}H]Met[^{14}C]Phe-tRNA^{Phe} bound in complex 1 after 10 min incubation in the respective buffer (triangles) and the maximum extent of f[^{3}H]Met[^{14}C]Phe-Pm formation after 10 min incubation with Pm (squares) were determined by nitrocellulose filtration and HPLC analysis, respectively.

(B) Plot of log (k_{pep}) vs. pH. Data from Figure 4A (closed circles). Values of k_{pep} calculated from the model of Figure 4D are also plotted (open circles). A theoretical curve calculated for a model with one ionizing group (slope 1.0) is shown for comparison (triangles).

(C) Base titration of Pm-HCl in unbuffered aqueous solution. A solution of 10 mM Pm-HCl in water was titrated with KOH at 37°C.

(D) Evaluation of titration data of Figure 4B. See text for details.

that the second ionizing group, protonation of which abolishes the reaction, has a pK_a of 6.9 ± 0.2. Assuming that Pm with a protonated amino group is inactive in the PT reaction, the pK_{a1} of 6.9 ± 0.2 might be attributable to Pm, consistent with the value of 6.9 ± 0.2 for the pK_a of free Pm obtained by titration (Figure 4C). If this assignment is correct, then the other ionizing group must be a ribosomal residue that titrates with a pK_a around 7.5.

A Single Ionizing Group of the Ribosome Is Involved in Catalysis

To test the assignment of pK_{a1} to the α-amino group of Pm, we used a derivative of Pm (Pm-OH) in which the amino group of phenylalanyl-3′-deoxy-3-amino-N, N-dimethyl adenosine was replaced with a hydroxyl group. Because the hydroxyl group is not protonated in the pH range studied here, Pm-OH can be used to study the pH profile of the PT reaction without interference by protonation of the nucleophile.

Due to the low water solubility of Pm-OH, assays had to be performed in the presence of 20% DMSO, but this had no effect on the rate of the reaction with Pm (data not shown). Compared to Pm, reaction rates with Pm-OH were much lower but exhibited the same concentration dependence (apparent K_d = 4 mM) (Figures 5A and 5B). The log(k_{pep}) versus pH plot (Figure 5C) revealed a pK_a of 7.5 ± 0.1 for the titrated group, similar to that seen with Pm. However, the slope of the plot was close to 1 (0.93 ± 0.05), suggesting that the rate of the PT reaction with Pm-OH was determined by a single ionizing group. This group must be ribosomal, because Pm-OH does not have a pK_a close to 7.5. The maximum rate of the reaction with Pm-OH was 0.1 s^{-1} (pH 8.2), in keeping with a lower nucleophilicity of the OH group compared to the NH_2 group of Pm. The strong dependence of the reaction rate on the nature of the nucleophile strongly supports the contention that the kinetic assay monitored the chemistry step of the reaction.

Inhibition of the PT Reaction by the A2451U Mutation in 23S rRNA

The effect of the substitution of A2451 with U was assessed using a ribosome preparation in which a fraction of the ribosomes carried the A2451U mutation (Thompson et al., 2001). With these ribosomes, time curves of the PT reaction with Pm were biphasic and had to be evaluated by double-exponential fitting (Figure 6A), whereas pure A2451 ribosomes with the same genetic background yielded strictly single-exponential curves (Figure 6A). At conditions of maximum rates, i.e., pH ≥

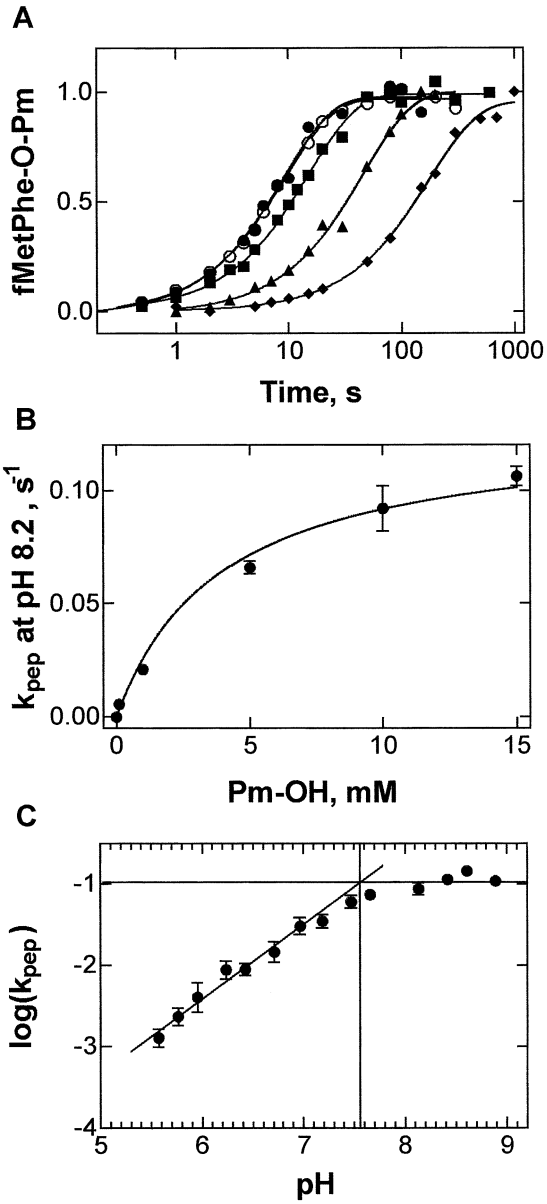

Figure 5. PT Kinetics with Pm-OH

(A) Time curves of fMetPhe-O-Pm formation. Quench-flow experiments were performed with complex 1 (Figure 1) in the presence of 20% DMSO at increasing concentration of Pm-OH (mM): 0.1 (diamonds), 1 (triangles), 5 (squares), 10 (closed circles), 15 (open circles). Single-exponential fitting (continuous lines) yielded k_{pep} values.

(B) Concentration dependence of k_{pep}. Fitting as in Figure 2 yielded $k_{pep} = 0.1\ s^{-1}$ (pH 8.2) at saturation and an apparent K_d of 4 mM.

(C) pH dependence of k_{pep}. Values for k_{pep} were measured at 10 mM Pm-OH at various pH values (cf. Figure 4) and are plotted as log(k_{pep}) versus pH. From the plot, the maximum value of $k_{pep} = 0.1\ s^{-1}$ and a slope of 0.93 ± 0.05 are derived.

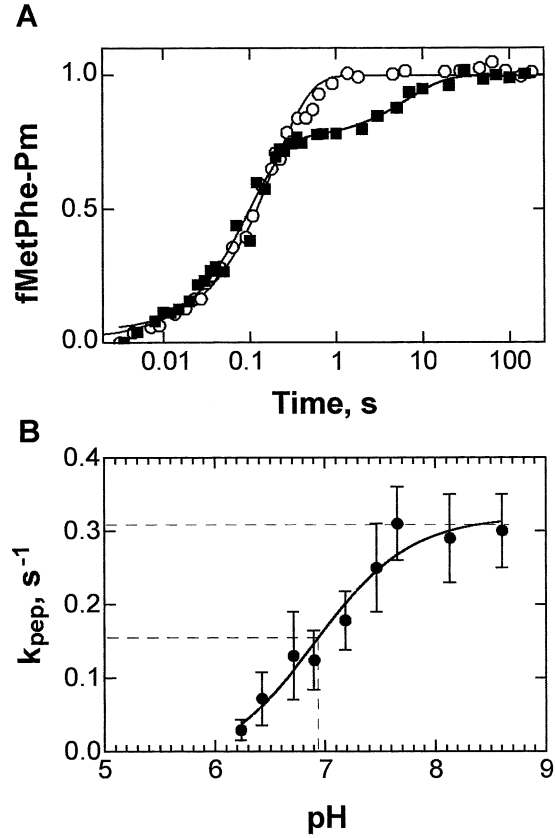

Figure 6. Influence of the A2451U Substitution on the Kinetics of the PT Reaction

(A) Time courses with the mixture of A2451 and U2451 ribosomes (closed symbols) and pure A2451 ribosomes (open symbols). All ribosome-bound f[^{3}H]Met[^{14}C]Phe-tRNA^{Phe} reacted with Pm. Double-exponential fitting of the curve obtained with the A2451 and U2451 mixture (pH 7.2) yielded two components for k_{pep}, a fast one with 12 s^{-1} (about 80% of the total amplitude) and a slow one with 0.18 s^{-1} (about 20% of the total amplitude), whereas a single exponential with $k_{pep} = 12\ s^{-1}$ was obtained with pure A2451 ribosomes.

(B) pH dependence of slow k_{pep} of mutant ribosomes. Fitting the data to a model with one ionizing group yielded $pK_a = 6.9$ and the maximum value of $k_{pep} = 0.3\ s^{-1}$ (continuous line).

7.7, the rate constant of the slow component was 0.3 s^{-1}. This was more than 100 times smaller than the fast component, 40 s^{-1}, which was about the same as that found for pure wild-type ribosomes. Thus, the slow PT reaction can be attributed to the fraction of mutant ribosomes present in the mixed preparation. This indicates that the A2451U substitution lowers the rate of the PT reaction about 130-fold. Previously reported activity reductions were smaller (5- to 14-fold), as measured with ribosomes containing 50S subunits with the A2451U substitution that were reconstituted in vitro (Polacek et al., 2001; Thompson et al., 2001). However, the reaction in those in vitro experiments was very slow (<2 min^{-1}), even with wild-type ribosomes, indicating that a step other than peptide bond formation was likely to be rate limiting in those assays.

The pH dependence of k_{pep} of the PT reaction by mutant ribosomes resulted in a slope of about 1 and a pK_a of 6.9 ± 0.2 (Figure 6B). This argues that a single ionizing group was involved in the reaction and that inhibition by protonation of a ribosomal group with $pK_a = 7.5$, as observed with wild-type ribosomes, was eliminated by the A2451U substitution. It appears that the inhibition observed upon lowering the pH with mutant ribosomes was due to protonation of Pm only.

Discussion

The rate of the uncatalyzed peptidyl transferase reaction is not known; it may be estimated to $<10^{-4}$ s^{-1}, based on rates observed in model reactions where aminolysis by amino acids of aminoacyl adenylates was studied (Weber and Orgel, 1979). As shown by the present kinetic analysis, the ribosome-catalyzed reaction between pept-tRNA and Pm proceeds at 50 s^{-1}, i.e., is accelerated more than 10^5-fold relative to the upper-limit estimate for the uncatalyzed reaction. This corresponds to a decrease of the free energy of activation by a total of >8 kcal/mol. The kinetic analysis has revealed that a ribosomal group with pK_a= 7.5 takes part in the reaction and that protonation of this group decreases the reaction rate about 100-fold (3 kcal/mol). This result is consistent with a contribution of general acid-base catalysis to overall catalysis. Alternatively, or in addition, protonation could induce a low-activity conformation of the active site. The residual reaction in the protonated state of the ribosome ($\sim$0.5 s^{-1}) is still >1000 times (>5 kcal/mol) faster than the uncatalyzed reaction, indicating that a major contribution to overall catalysis comes from positional effects, i.e., the binding of the 3′ terminus of pept-tRNA and of Pm (or aa-tRNA) by base pairing to residues in the P and A loops of 23S rRNA in the PT center.

On the basis of the crystal structure, it has been proposed that the group accepting the proton in the PT reaction might be N3 of A2451, which is located within 3–4 Å of the nucleophilic amino group to be deprotonated upon formation of the tetrahedral intermediate (Nissen et al., 2000). The strong inhibition of the PT reaction resulting from the A2451U substitution, which also eliminates the pK_a of the ribosomal group, would be in line with the proposed mechanism. However, as the unperturbed pK_a of N3 is expected to be significantly lower than that of N1, which is around 3.5, the mechanism requires that the pK_a be shifted very substantially, and it is uncertain whether the proposed charge relay system involving G2447 (Nissen et al., 2000) could bring about such a large perturbation. Furthermore, exchanging G2447 for other bases had only small effects on PT activity (Polacek et al., 2001; Thompson et al., 2001). Although these arguments do not necessarily exclude that protonation of A2451 is involved in the PT reaction, one may consider alternative ionizing groups in the active site. One attractive alternative is the wobble base pair A2450-C2063 that flanks A2451 (Muth et al., 2001). The formation of A-C wobble pairs requires protonation of N1 of adenine, resulting in a pK_a shift from 3.8 (unperturbed) to values approaching neutrality, as shown by NMR (Cai and Tinoco, 1996). In the 50S crystal structure, N1 of A2450 is about 7 Å apart from the amide nitrogen of CCdA-p-Pm bound in the active site as an analog of the tetrahedral reaction intermediate (Nissen et al., 2000), i.e., too far away for direct proton transfer. However, it is conceivable that, during formation of transition state 1 (Hegazi et al., 1978), the two groups come together close enough to allow proton transfer from the nucleophilic amino group to N1 of A2450 with the subsequent formation of the A-C base pair that signifies the state with the tetrahedral intermediate. During breakdown of the intermediate via transition state 2, the proton could be donated to the leaving ribose 3′-O group to form the 3′-OH of the P site product while the A-C pair is resolved.

Formation and resolution of an A-C base pair may provide an additional contribution to catalysis by inducing conformational changes of the active site that favor the progress of the reaction. In fact, there are strong indications for pH-dependent conformational effects. The pH-sensitive accessibility of A2451 for chemical modification (Muth et al., 2000) was attributed to conformational changes of the 50S subunit (Bayfield et al., 2001), and several bases within the PT center of ribosomes from various organisms, including *E. coli*, exhibited pH-dependent variations of accessibility for chemical modification, indicating conformational flexibility within the PT center (Muth et al., 2001; Xiong et al., 2001). The A2450-C2063 base pair discussed above, and a second A-C pair (A2453-C2499) located next to it, may be involved in these structural changes (Muth et al., 2001).

How to explain the slow reaction and the loss of pH sensitivity observed with A2451U ribosomes, if A2451 were not the base to be protonated? Based on the crystal structure of 50S with CC-Pm bound (Nissen et al., 2000), N3 of A2451 accepts an H-bond from the NH_2 group of CC-Pm (Muth et al., 2001), which may promote the reaction by positioning the nucleophilic amino group. The strong inhibition caused by the A2451U mutation then would indicate that O2 of U2451 does not participate in such an H-bond. Furthermore, the A2451U substitution may cause a structural perturbation in the active site, such that the formation of the A2450-C2063 pair discussed above, where A2450 is stacked onto A2451, is no longer favored.

In conclusion, the kinetic dissection of the PT reaction suggests that catalysis by the ribosome comprises both positional and general acid-base catalysis. Clearly, further mutational and structural work is required to substantiate this mechanism. Furthermore, it will be interesting to study the mechanism with the true A site substrate, aa-tRNA, instead of Pm, under conditions where its accommodation in the active site is no longer rate limiting.

Experimental Procedures

Preparation of Ribosome Complexes

Materials were prepared as previously described (Rodnina and Wintermeyer, 1995). Ribosomes bearing the A2451U mutation in 23S rRNA were isolated from cells containing a plasmid coding for 23S rRNA under temperature-sensitive transcription control (Thompson et al., 2001), purified as described (Rodnina and Wintermeyer, 1995), and used for the preparation of posttranslocation complex 1 (Figure 1). The following buffers were used: pH 5.2–5.5, MES/Tris-HCl (20/50 mM); pH 5.5–8.5, Bis-Tris/Tris-HCl (20/50 mM); pH 8.5–9.0 glycine/Tris-HCl (20/50 mM).

The 70S initation complex with f[^{3}H]Met-tRNA^{fMet} in the P site was prepared in buffer A (50 mM Tris-HCl [pH 7.2], 30 mM KCl, 70 mM NH_4Cl, and 7 mM $MgCl_2$) by incubating ribosomes (1 μM; isolated from *E. coli* MRE600) with MFT-mRNA (4 μM; coding for fMetPheThr) and f[^{3}H]Met-tRNA^{fMet} (1.5 μM; 4000 dpm/pmol) in the presence of initiation factors 1, 2, and 3 (1.5 μM each) for 15 min at 37°C. Binding of EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe} (1.0 μM; 1000 dpm/pmol) and peptide bond formation resulted in ribosomes carrying deacylated tRNA^{fMet} in the P site and f[^{3}H]Met[^{14}C]Phe-tRNA^{Phe} in the A site. Upon incubation (5 min) with EF-G (0.02 μM) and GTP (1 mM),

f[^{3}H]Met[^{14}C]Phe-tRNA^{Phe} was translocated to the P site. All partial reactions proceeded with quantitative yield, resulting in ribosome complexes that were >90% occupied with f[^{3}H]Met[^{14}C]Phe-tRNA^{Phe} in the P site. The ribosomes containing f[^{3}H]Met[^{14}C]Phe[^{14}C]Phe in the P site (Figure 3) were prepared as in Figure 1, except that MFF-mRNA (coding for fMetPhePhe) and 2.5 μM of EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe} were used; those containing an olipeptidyl-tRNA as in Figure 1, except that an mRNA coding for fMetAlaAsnMetPheAlaLeu was used and the synthesis of the oligopeptide was carried out with a mixture of the EF-Tu·GTP·aa-tRNA complexes that were prepared from unfractionated *E. coli* tRNA charged with unlabeled Asn, Met, Phe, and [^{14}C]Ala and purified by gel filtration on Superdex 75.

Complexes were purified and concentrated by centrifugation through 1.1 M sucrose cushions at 200,000 × g for 1.5 hr (Sorvall M120GX). Ribosome pellets were redissolved in buffer A to about 4 μM concentration, quick-frozen in liquid nitrogen, and stored at –80°C. For the kinetic assays, ribosome stocks were diluted 1:10 into the desired buffer, and the pH of both ribosome and Pm solutions was measured using a small-size pH electrode. Quench-flow assays were performed at 37°C in a KinTek apparatus, mixing equal volumes (12 μl) each of ribosome and Pm solution. Reactions were quenched with KOH (0.5 M), and f[^{3}H]Met[^{14}C]Phe-Pm formed by the PT reaction and unreacted f[^{3}H]Met[^{14}C]Phe, set free from f[^{3}H]Met[^{14}C]Phe-tRNA^{Phe} by alkaline hydrolysis (30 min, 37°C), were separated by RP-HPLC[17] and quantitated by double-label radioactivity counting. Reactions with PM-OH were stopped by the addition of 25% formic acid, and f[^{3}H]Met[^{14}C]Phe-O-Pm in the supernatant was analyzed by RP-HPLC.

Synthesis of Pm-OH

Pyridine (0.053 ml, 0.66 mmole), chlorotrimethylsilane (0.084 ml, 0.66 mmole), and catalytic dimethylamino pyridine were added to a solution of L-3-phenyllactic acid (50 mg, 0.3 mmole) in anhydrous methylene chloride. The reaction was stirred at room temperature for 4 hr and then cooled to 0°C, at which time oxalyl chloride (0.029 ml, 0.33 mmole) was added followed by catalytic N,N dimethyl formamide. After 30 min at room temperature, 3′-amino-3′deoxy-N,N-dimethyladenosine (30 mg, 0.1 mmole) in anhydrous pyridine was added (Kelly and LaCour, 1992). The reaction was quenched after 10 min with 50% NH_4OH/MeOH. Chromatographic purification on silica (5% MeOH/CH2Cl2) yielded 17 mg (38%) of a white solid. 1H NMR δ 1.63 (s, 6H), 2.93 (q, 1H), 3.23 (dd, 1H), 3.81 (m, 1H), 3.96 (m, 1H), 4.22 (m, 1H), 4.39 (m, 1H), 4.46 (m, 1H), 4.77 (m, 1H), 5.21 (m, 1H), 5.76 (d, 1H), 7.33 (m, 5H), 7.96 (s, 1H), 8.24 (s, 1H); MS calcd. for C21H27N6O5+: 443.2043, found M+H 443.2041.

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