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Peptide bond formation on the ribosome: structure and mechanism

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The peptidyl transferase reaction on the ribosome is catalyzed by RNA. Pre-steady-state kinetic studies using *Escherichia coli* ribosomes suggest that catalysis (>10^5-fold overall acceleration) is, to a large part, a result of substrate positioning, in agreement with crystal structures of large ribosomal subunits with bound substrate or product analogs. The rate of peptide bond formation is inhibited approximately 100-fold by protonation of a single ribosomal group with a pK_a of 7.5, indicating general acid-base catalysis and/or a pH-dependent conformational change within the active site. According to the kinetics of mutant ribosomes, these effects may be attributed to a candidate catalytic base (A2451) suggested by the crystal structure.

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Current Opinion in Structural Biology 2003, **13**:334–340

This review comes from a themed issue on
Nucleic acids
Edited by Carl C Correll and David MJ Lilley

0959-440X/03/$ – see front matter

DOI 10.1016/S0959-440X(03)00065-4

Abbreviations

aa-tRNA	aminoacyl-tRNA
pept-tRNA	peptidyl-tRNA
Pm	puromycin
PT	peptidyl transferase

Introduction

Ribosomes synthesize proteins from activated aminoacyl esters of tRNAs (aa-tRNAs). During aa-tRNA binding and translocation in the protein elongation cycle, the ribosome is assisted by accessory enzymes, called elongation factors. Peptide bond formation is catalyzed by the ribosome itself at a rate >10 s^{-1}. The rate of the uncatalyzed reaction has been estimated to be <10^{-4} s^{-1}, based on rates observed in model reactions of the aminolysis of aminoacyl adenylates by amino acids [1]. Thus, the ribosome accelerates the reaction at least 10^5-fold.

The active site for peptide bond formation, the peptidyl transferase (PT) center, is located on the large ribosomal subunit. 50S subunits that were largely depleted of proteins still exhibited PT activity, indicating that 23S rRNA had an important role in catalysis [2]. However, a contribution from ribosomal proteins could not be excluded completely [3,4] and protein-free 23S rRNA that promotes peptide formation has not been obtained so far. The recent determination of high-resolution crystal structures of the large ribosomal subunit has revealed that the PT center, as localized by an intermediate-state analog, is composed of RNA (23S rRNA) [5••,6••]. This implies that the PT reaction is catalyzed by RNA.

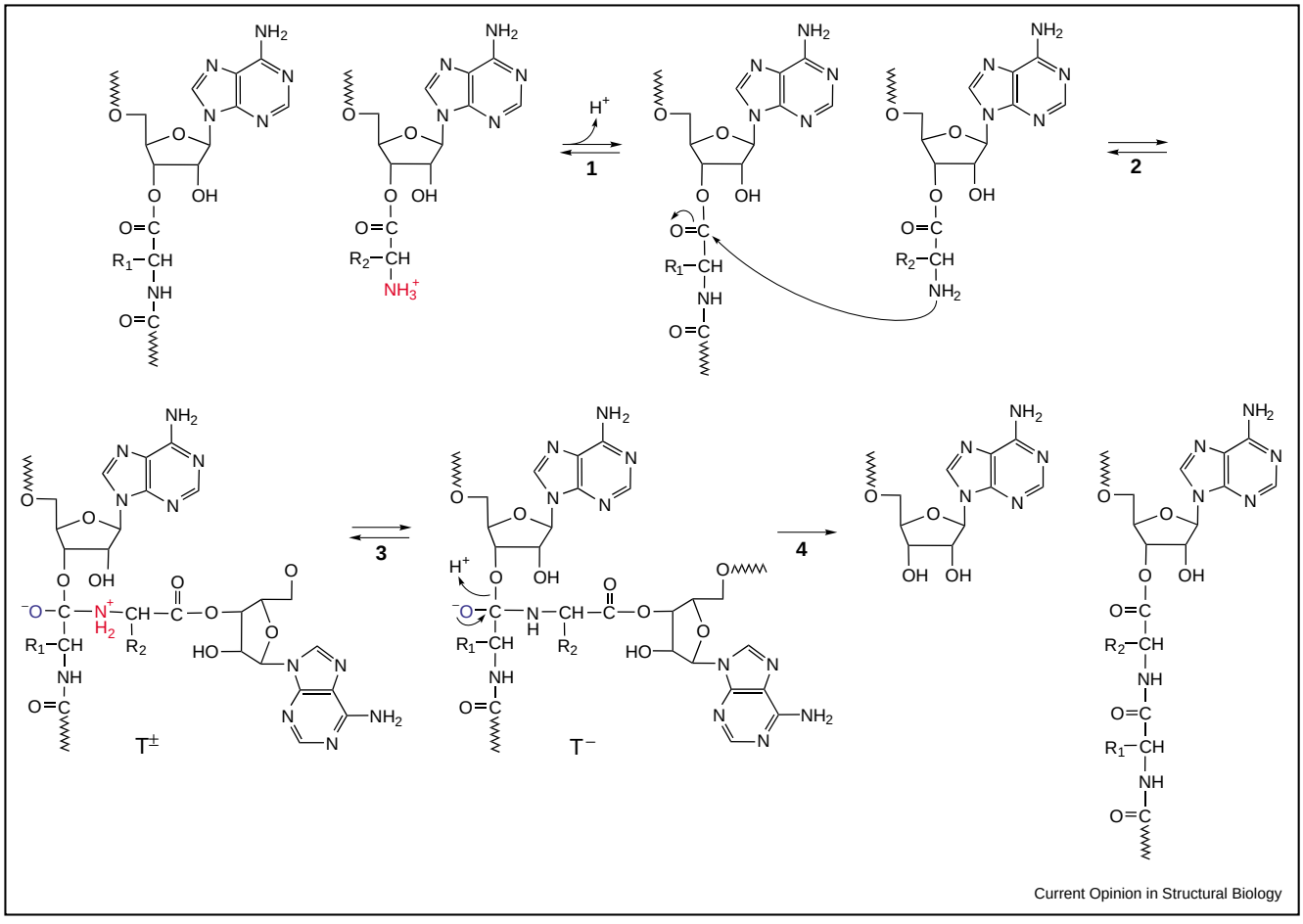
Naturally occurring catalytic RNAs (ribozymes) catalyze phosphoryl transfer reactions that require the activation of a 2′-OH group (nucleolytic ribozymes) or a water molecule (RNase P) for nucleophilic attack on a neighboring phosphodiester bond (see [7] for a review). The ribosome constitutes the first example of a natural ribozyme with (amino acid) polymerase activity. A synthetic ribozyme exhibiting low PT activity was obtained by *in vitro* selection [8].

Recently, a mechanism of catalysis was proposed based on the arrangement of RNA elements in the active site and their interactions with an intermediate-state analog [6••]. In this model, an important catalytic function was attributed to one particular residue in the active site, A2486 (*Thermus thermophilus*; A2451 in *Escherichia coli*; *E. coli* numbering is used in this review). The proposal has prompted genetic, biochemical and kinetic work, a summary of which is the main focus of this review. An excellent recent minireview is also available [9••]. Earlier work has been reviewed previously [10–13].

Chemistry of peptide bond formation

The peptide bond is formed by nucleophilic attack on the ester carbonyl group of peptidyl-tRNA (pept-tRNA) bound to the P-site by the α-amino group of aa-tRNA in the A-site of the PT center (Figure 1). The first step is the deprotonation of the α-NH_3^+ group to create the nucleophilic NH_2 group (step 1, Figure 1). The pK_a of this group in aa-tRNA is estimated to be around 8 and it is likely that the proton is accepted by water. Subsequent nucleophilic attack of the α-NH_2 group on the electrophilic carbonyl group (step 2, Figure 1) leads to the formation of the initial protonated tetrahedral reaction product, $T^{\pm}$, which, by deprotonation (step 3), forms the tetrahedral intermediate, T^-. Steps 2 and 3 can also take place synchronously, as has been suggested for the PT reaction [6••], that is, $T^{\pm}$ is not a necessary reaction intermediate. Breakdown of T^- (step 4) is initiated by donating a proton back to the leaving oxygen to form the products: P-site deacylated tRNA and A-site pept-tRNA.

Figure 1



The chemistry of peptide bond formation. The 3′ ends of P-site pept-tRNA (left) and A-site aa-tRNA (right) are depicted. Step 1: deprotonation of the NH_3^+ group. Step 2: nucleophilic attack of the NH_2 group on the ester carbonyl group and formation of a zwitterionic tetrahedral intermediate, $T^{\pm}$ [33]. Step 3: deprotonation and formation of the negatively charged tetrahedral intermediate, T^-. Step 4: product formation by breakdown of the tetrahedral intermediate upon protonation of the leaving oxygen. Steps 2 and 3 can also take place synchronously. Products are P-site deacylated tRNA (left) and A-site pept-tRNA (right). R_1 and R_2, amino acid sidechains. Red and blue, groups to be deprotonated or protonated, respectively.

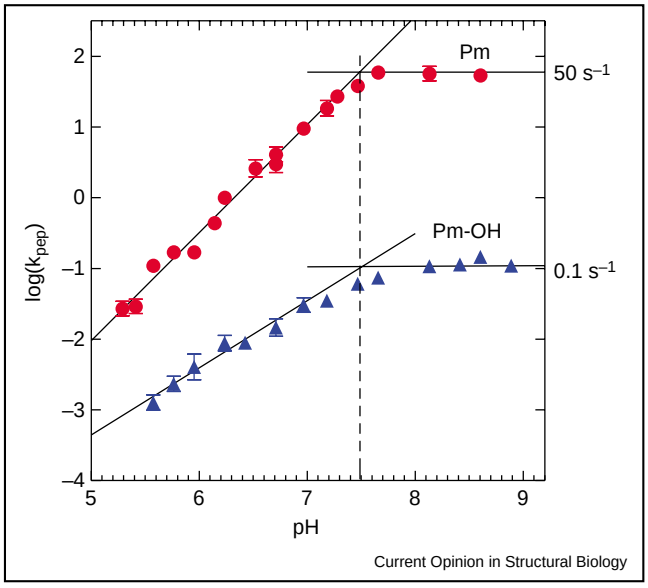
Catalysis of the PT reaction could involve any of the following mechanisms: proper positioning of the peptidyl and aminoacyl ends of the tRNAs in the active site; general acid-base catalysis during deprotonation (step 3, Figure 1) and protonation (step 4); electrostatic stabilization of the transition state(s) leading to $T^{\pm}$ or T^-.

pH dependence of peptide bond formation

Early studies revealed that the PT reaction is strongly pH dependent [14,15]. In addition to inhibition by protonation of the nucleophilic NH_2 group, the reaction was also inhibited by protonation of a group (or groups) on the ribosome with a pK_a around 7.5 [16]. Although these results indicated that the reaction might involve general acid-base catalysis, the shortcoming of the early experiments was that reaction rates were quite low, in the minute range, suggesting that the observed rates were limited by some step(s) other than the chemical step.

This limitation was overcome in a recent study in which the PT reaction was studied under conditions in which the chemical step was rate limiting; rates up to 50 s^{-1} were attained using the quench-flow technique [17••]. In these experiments, fully active ribosomes programmed with natural mRNA and carrying dipeptidyl-tRNA (fMetPhe-$tRNA^{Phe}$) in the P-site were reacted with puromycin (Pm) as the A-site substrate. Pm, rather than aa-tRNA, was used because peptide bond formation between pept-tRNA and aa-tRNA, which is intrinsically rapid, is rate limited by the preceding step of aa-tRNA accommodation in the PT center (rate constant about 10 s^{-1}; [18]). At sufficiently high Pm concentration ($\geq$10 mM), the reaction rate was no longer dependent on Pm concentration (i.e. not limited by Pm binding). This system was used to monitor the rate of the chemical step of the PT reaction between dipeptidyl-tRNA and Pm, and to measure the pH dependence of the reaction.

Figure 2



The pH dependence of the PT reaction. Ribosomes with dipeptidyl-tRNA in the P-site were reacted with Pm or Pm-OH, and rate constants (k_{pep}) of peptide bond formation were determined at different pH. The slope of the log(k_{pep}) versus pH plot is 1.5 for Pm; fitting the data to a model with two ionizing groups yielded $k_{pep} = 0.5\ s^{-1}$ for the singly protonated system ($pK_a = 7.5$) and $k_{pep} < 0.01\ s^{-1}$ for the doubly protonated system ($pK_a = 6.9$). For Pm-OH, the slope is close to 1.0 and the ionizing group has a pK_a of 7.5.

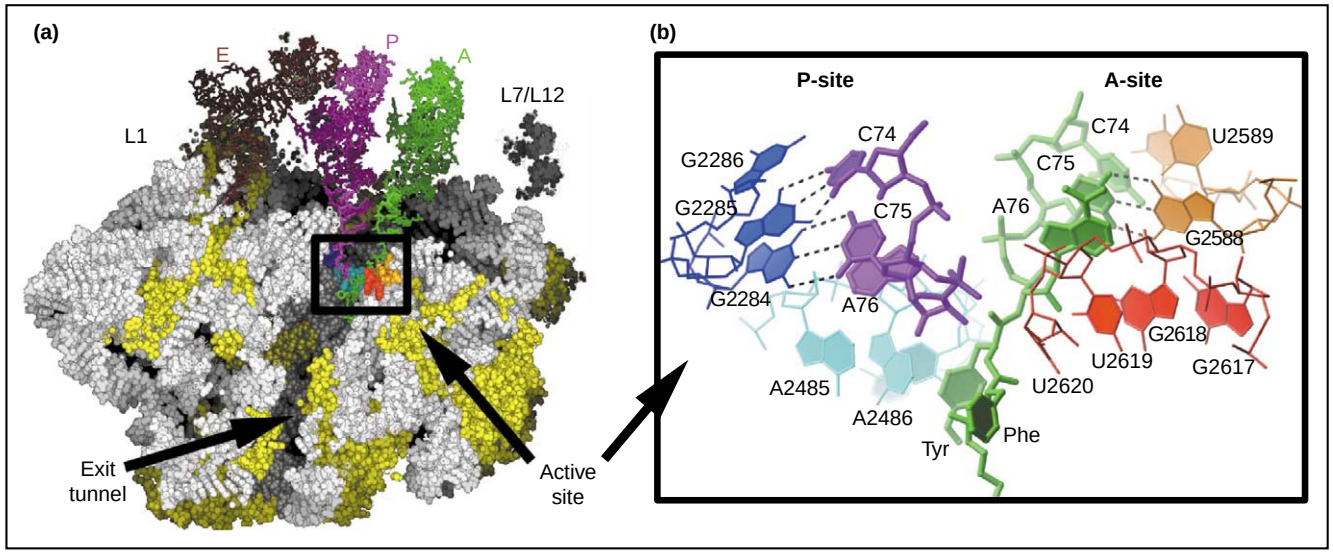
The reaction rate was strongly pH dependent (Figure 2). This effect is not due to ribosome inactivation, because the extent of the reaction remained close to 100% over the entire pH range studied. The plot of log(k_{pep}) versus pH had a slope of 1.5, indicating that more than one ionizing group was involved in the reaction. At pH values >7.7, the maximum reaction rate of $50 \pm 10\ s^{-1}$ was reached and the highest pK_a value of the ionizing groups, $pK_a = 7.5 \pm 0.1$, was determined from the plot directly. Fitting the data to a model with two ionizing groups revealed a second pK_a value of 6.9 and a reaction rate for the singly protonated system (at the group with a pK_a of 7.5) of $0.5\ s^{-1}$, approximately 100-fold lower compared to the fully deprotonated system. The pK_a value of 6.9 was assigned to the NH_2 group of Pm, as determined by titration, and protonation of this group virtually eliminated the reaction. The assignment was confirmed by experiments with a Pm derivative, Pm-OH, in which the nucleophilic amino group was replaced with a hydroxyl group (Figure 2). In keeping with the lower nucleophilicity of OH compared to NH_2, the PT reaction with Pm-OH was rather slow ($k_{pep} = 0.1\ s^{-1}$ at high pH), supporting the notion that the chemical step was monitored in these assays. Interestingly, the slope of the log(k_{pep}) versus pH plot with Pm-OH was 1.0 and the pK_a of the single ionizing group was 7.5. Thus, the pK_a of 7.5 must be attributed to a ribosomal group, as Pm-OH does not have a pK_a of around 7.5.

There are two important conclusions from these results. One is that, in the singly protonated state, that is, when the ribosomal group with a pK_a of 7.5 is protonated, the reaction is still >1000-fold faster than the uncatalyzed reaction. This part of overall catalysis may be attributed to substrate positioning, proximity effects and electrostatic stabilization of the transition state. The second conclusion is that the pH-sensitive part of the reaction, which is attributed to protonation of a single ribosomal group with a pK_a of 7.5, contributes another factor of 100 to the overall rate. This could be due to general acid-base catalysis. Alternatively, protonation could induce a conformation of the active site in which the approach to the transition state is impaired for structural reasons. In such a case, mainly positional and related effects would be responsible for overall catalysis. In fact, there are strong indications of pH-dependent conformational changes in the active site [19–22]. It is important to note that the approximately 100-fold inhibition of the PT reaction by protonation, observed in the kinetic experiments, is a rate effect and is not due to inactivation of the ribosome, as the ribosomes retain their ability to form peptide. Thus, the effect has to be distinguished from ribosome inactivation, which was reported to result in similar structural alterations to changes of pH [19].

Structure of the active site: substrate positioning and potential catalytic residues

The atomic structure of the large ribosomal subunit from *Haloarcula marismortui* shows that the PT center, delineated by the tetrahedral intermediate analog CCdA-phosphate-puromycin (CCdApPm) bound to the active site, is composed of RNA [5••,6••]. A similar active site structure was observed in 50S subunits from *Deinococcus radiodurans* [23•]. Several base pairs between the CCA end sequences of substrate or product analogs and bases in the P- and A-loops of the active site were revealed by the 50S crystal structures [6••,24••,25•]. In the P-site, C74 and C75 are paired to G2252 and G2251 (*E. coli* numbering) of the P-loop and, in the A-site, C75 is paired to G2553 in the A-loop (Figure 3); these base pairs are essential for the PT reaction [26,27]. The functional relevance of the model complex structures was suggested by the demonstration of PT activity by 50S crystals [24••]. Additionally, in both sites, A76 residues are involved in A-minor interactions with 23S rRNA [6••]. Comparison with the structure of 70S ribosomes with bound tRNAs [28••] suggests that the CCA ends of full-size tRNA substrates are arranged in the active site in a similar fashion. Crystal structures of 50S subunits from *D. radiodurans* with small substrate analogs revealed a similar arrangement in the active site [29•], although a larger acceptor minihelix coupled to Pm as the A-site substrate analog was found to adopt a somewhat different arrangement compared to the

Figure 3



Interactions of tRNA CCA ends in the PT center. **(a)** Cut-away view of the large ribosomal subunit. tRNA molecules in the A- (green), P- (purple) and E- (brown) sites are modeled from the 70S crystal structure [28••]. **(b)** Base-pairing interactions of CCA sequences with 23S rRNA. In the P-site, CCA (purple), an analog of deacylated tRNA, is bound to bases of the P-loop (blue). The analog of pept-tRNA in the A-site (green), a derivative of CCA-Pm-Phe, is bound to bases of the A-loop (brown). Bases are numbered according to the *H. marismortui* 23S rRNA sequence. Potential ionizing bases (light blue) discussed in the text are A2486 and A2485 (A2451 and A2450 in *E. coli*). Figure reproduced from [24••] with permission.

respective *H. marismortui* complex structure [25•]. The differences were interpreted to indicate that details of the arrangement of the CCA termini in the active site are influenced by interactions of the tRNA molecule with ribosomal elements outside the active site [29•].

Although crystal structures and biochemical evidence indicated the importance of positional catalysis, the pH dependence of the PT reaction suggested there might be an additional contribution from general acid-base catalysis. In the crystal structures, there was no evidence of the presence of catalytic metal ions, leaving 23S rRNA bases as potential catalytic residues. Based on the crystal structure, a mechanism for the PT reaction was proposed that involved general acid-base catalysis by 23S rRNA residues [6••]. N3 of a conserved adenine (A2486 in *H. marismortui*; A2451 in *E. coli*) was suggested to act as the catalytic base, which, upon approach to the transition state, abstracts a proton from the NH_2 group and, during subsequent breakdown of the tetrahedral intermediate, donates it back to the leaving oxygen (Figure 1). It is generally assumed that the catalytic base under physiological conditions needs to have a pK_a around neutrality. In this case, the pK_a of the NH_2 group to be deprotonated is very high in the ground state (>30), but will decrease by acquiring a positive charge upon approaching the transition state (Figure 1). It is conceivable, therefore, that a group exhibiting the experimentally observed pK_a of 7.5 will abstract the proton in the transition state or a state close to it. One potential proton acceptor is N3 of A2451 [6••], in particular because it seems to be hydrogen bonded to the nucleophilic NH_2 group [6••,25•]. However, unperturbed pK_a values for adenine are 3.5 (N1) or lower (N3), and it is uncertain whether the proposed charge relay system involving G2447 and other residues [6••] could bring about the large perturbation that is required for efficient proton transfer.

Alternative ionizing groups in the active site may be considered to explain the observed pH dependence. An attractive candidate is A2450, which flanks A2451 and is base paired to C2063 in the crystal [30]. The formation of A•C base pairs requires protonation of N1 of adenine, resulting in a pK_a shift from 3.8 (unperturbed) to values approaching neutrality [31]. Thus, the A2450•C2063 base pair and a second A•C pair (A2453•C2499) located next to it may well exhibit a pK_a around 7.5, and the observed inhibition of the PT reaction could be attributed to protonation of A2450. Whether A2450 could function as a catalytic residue depends on its steric arrangement. For direct proton transfer, N1 would have to be within hydrogen-bonding distance (~3 Å) of the NH_2^+ group to be deprotonated upon forming the tetrahedral intermediate. In the 50S crystal structure with CCdApPm in the active site, this distance is approximately 7 Å; however, in this structure, the A2450•C2063 base pair is present, that is, N1 of A2450 must be protonated. The conformational change of 3–4 Å required for A2450 to take part in the reaction directly appears possible, in particular as we are extrapolating from the crystal structure of isolated 50S subunits

with small substrate or product analogs to 70S ribosomes with full-length tRNA substrates.

Effects of base exchange in the active site

The functional consequences of exchanging the bases at positions 2451 and 2447, which have both been implicated in the proposed mechanism of peptide bond formation [32], were studied *in vivo* and *in vitro*. Mutating A2451 in *E. coli* conferred a dominant-lethal phenotype [20,21,32], consistent with a critical role for A2451 in peptide bond formation. In keeping with the *in vivo* results, kinetic experiments have revealed that the rate of peptide bond formation is 130-fold lower and pH independent on ribosomes carrying an A2451U mutation [17••]. Smaller effects *in vitro* reported by other groups [20,21] probably reflect the fact that, in these experiments, the chemical step was not rate limiting.

E. coli cells containing only ribosomes with a G2447A mutation are viable [20], indicating that guanine at position 2447 is nonessential, and two groups have reported only small effects *in vitro* caused by this mutation [20,21]. By contrast, preliminary results from rapid kinetic experiments show that the G2447A mutation in *E. coli* ribosomes has a significant inhibitory effect on the chemical step of the PT reaction (M Beringer, S Adio, W Wintermeyer, MV Rodnina, unpublished data).

These results are consistent with the proposed role of A2451, assisted by G2447 and other nearby residues, in acid-base catalysis of peptide bond formation ([6••]; see also Update). However, the extensive pK_a shift of A2451, required by the model, has not been shown experimentally. In an alternative model, assuming A2450 is the group ionizing at pK_a 7.5 [17••], it is not immediately obvious why the A2451U mutation would eliminate the inhibition by A2450 protonation assumed by this model. A plausible explanation would be that A2450 is stacked directly onto A2451 and that the loss of this stacking interaction as a consequence of the A2451U mutation changes the position of A2450 such that the formation of the A•C base pair and, with that, protonation of N1 of A2450 would no longer be favored energetically.

Conclusions

The results of mechanistic and structural studies greatly enhance our understanding of the catalytic mechanism of peptide bond formation. The combined evidence strongly supports the idea that substrate positioning provides a major contribution to catalysis. The main supporting observations from structural analysis are the precise alignment of A- and P-site substrates through interactions of their CCA sequences, and of the nucleophilic amino group of the A-site substrate with residues of 23S rRNA in the active site. Interfering with these interactions by mutating 23S rRNA residues impairs the reaction. The quantitative analysis, by kinetic dissection of the reaction into pH-dependent and pH-independent parts, indicates that positional catalysis accelerates the reaction >1000-fold relative to the uncatalyzed reaction.

The approximately 100-fold inhibition of the PT reaction by protonation of a ribosomal residue with a pK_a of 7.5 may indicate a contribution to overall catalysis from a general acid-base mechanism. Candidate catalytic residues, including A2451 and A2450 (in *E. coli* 23S rRNA), are suggested by the crystal structures. The inhibition of the PT reaction resulting from mutating A2451 is in line with such a mechanism. However, the results obtained up to now would also be compatible with the induction, by protonation of a ribosomal group, of a low-activity conformation of the active site.

Clearly, at this point we have insufficient data to make any decision between these two possibilities or to qualify their relative contributions. More 23S rRNA mutants will have to be studied both structurally and kinetically. However, this approach has its limitations, because introducing changes in the active site can have structural effects unrelated to the catalytic mechanism that may affect the reaction. Another important issue concerning future work comes from the fact that the structural and mechanistic work up to now was performed with model compounds replacing substrates and products. Thus, it will be interesting (and important) to study the mechanism of peptide bond formation with full-size aa-tRNA as the A-site substrate under conditions in which its accommodation in the active site is no longer rate limiting. These are difficult experiments and we still have a long way to go until we get a more complete picture of the catalytic mechanism employed by the ribosome to bring about rapid peptide bond formation.

Update

Recent work has shown that the G2447A mutation in 23S rRNA of *E. coli* ribosomes decreases the rate of peptide bond formation between P-site pept-tRNA and A-site Pm about tenfold, and does not eliminate the pH dependence of the reaction that is attributed to a ribosomal group with a pK_a of about 7.5 [34]. This finding does not support the charge relay mechanism involving G2447 that was proposed to shift the pK_a of A2451 to the extent required for the function of A2451 in general acid-base catalysis [6••].

Acknowledgements

The work on the kinetics of peptide bond formation has, to a large part, been carried out by VI Katunin, Russian Academy of Sciences. We acknowledge cooperation with SA Strobel and GW Muth, Yale University. Work in our laboratories is supported by the Deutsche Forschungsgemeinschaft, the EC-Program Quality of Life (contract QLK2-CT-2002-00892), the Alfried Krupp von Bohlen und Halbach-Stiftung and the Fonds der Chemischen Industrie.

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Crystal structures of *D. radiodurans* 50S crystals with substrate analogs bound to A- or P-sites are analyzed. A small analog (CCA-Pm) is arranged

in the A-site in a similar fashion as in *H. marismortui* 50S [25•]. An extended minihelix coupled to Pm binds differently and does not seem to approach A2451. It is proposed that the arrangement of the 3′ tRNA termini in the active site is influenced by interactions between the rest of the tRNA and the ribosome (e.g. protein L16, helix 69 of 23S rRNA).

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Now in press

The work referred to in the text as (M Beringer, S Adio, W Wintermeyer, MV Rodnina, unpublished data) is now in press:

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