Peptide Release on the Ribosome Involves Substrate-Assisted Base Catalysis

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Supporting Information

ABSTRACT: Termination of protein synthesis on the ribosome involves hydrolysis of the ester bond between the P-site tRNA and the nascent peptide chain. This reaction occurs in the peptidyl transferase center and is triggered by the class I release factors RF1 and RF2 in prokaryotes. Peptidyl-tRNA hydrolysis is pH-dependent, and experimental results suggest that an ionizable group with pKₐ > 9 is involved in the reaction. The nature of this group is, however, unknown. To resolve this problem, we conducted density functional theory calculations using a large cluster model of the peptidyl transferase center. Our calculations reveal that peptidyl-tRNA hydrolysis occurs via a base-catalyzed mechanism with a predicted activation energy of 15.8 kcal mol⁻¹, which is in good agreement with experimental data. In this mechanism, the P-site A76 2′-OH group is deprotonated and acts as the general base by activating the nucleophilic water molecule. The energy cost of deprotonating the 2′-hydroxyl group at pH 7.5 is estimated to be about 8 kcal mol⁻¹, on the basis of its experimental pKₐ in aqueous solution, and this step is predicted to be the source of the observed pH dependence. The proposed mechanism is consistent not only with experimentally derived activation energies but also with the observed kinetic solvent isotope effect.

KEYWORDS: ribosome, translation termination, release factor, peptidyl-tRNA hydrolysis, density functional theory

INTRODUCTION

Termination of mRNA translation on the ribosome occurs when a stop codon is presented in the A-site of the ribosomal decoding center. In bacteria, the three stop codons are recognized by two class I release factors (RFs), where RF1 reads UAA and UAG, while RF2 reads the UAA and UGA codons. Binding of the RFs to the decoding center triggers hydrolysis of the ester bond between the newly synthesized polypeptide and the last tRNA, residing in the P-site of the ribosome. This reaction takes place in the peptidyl transferase center (PTC), located some 75 Å away from the decoding site, and is stimulated by the universally conserved GGQ motif in domain 3 of the RFs. The GGQ loop inserts the glutamine side chain into the PTC, where it positions a water molecule for nucleophilic attack on the ester carbonyl carbon, and the glutamine backbone also stabilizes the developing negative charge on the ester carbonyl oxygen.1–5 The PTC catalyzes two distinct reactions (Figure 1): namely, peptide bond formation (ester aminolysis) during the elongation phase of protein synthesis and peptidyl-tRNA hydrolysis (ester hydrolysis) in the termination phase. Both of these reactions thus involve cleavage of the P-site peptidyl-tRNA ester bond to the 3′-oxygen of A76, but with different nucleophiles. Nucleophilic attack of the α-amino group of the A-site aminoacyl-tRNA on the ester carbonyl results in peptidyl transfer, while attack of water at the same position results in translation termination. Density functional theory (DFT) calculations predicted that the rate-limiting step for peptide bond formation is breaking of the C–O bond in an eight-membered transition state (TS) involving a double proton shuttle.6 This transition state is consistent with both the pH-rate profile7 and the large observed kinetic solvent isotope effect (KSIE) of 8.2 for peptide bond formation.8,9

By analogy, it would thus seem reasonable to assume that peptidyl-tRNA hydrolysis also proceeds by the same mechanism. However, recent experimental results seem to contradict such a view. First, the pH dependence of the hydrolysis reaction...
is distinctly different from that of peptide bond formation, which may be interpreted such that hydroxide ion rather than neutral water is the nucleophile.\textsuperscript{9,10} Such a mechanism was, in fact, modeled in earlier computer simulations of the termination reaction with a dA⁷⁶ peptidyl-tRNA substrate, where very similar relative rates were found for various RF1 mutants in comparison to the neutral water mechanism.\textsuperscript{10} If the observed pH dependence does not reflect ionization of either the water nucleophile or the 2′-OH group of A⁷⁶, the reaction would have to be dependent on deprotonation of some other ribosomal group, but there is no obvious candidate. Moreover, the pK\textsubscript{a} in water of the 2′-OH group in 3′-O-methyladenosine\textsuperscript{12} has been shown to be as low as 13.7, which could make this group a viable candidate for ionization and base catalysis. Moreover, proton inventory experiments and the measured KSIE value of 4.1 for the hydrolysis reaction appear to be inconsistent with an eight-membered TS.\textsuperscript{9} Hence, the above experimental observations clearly suggest a change of mechanism for peptidyl-tRNA hydrolysis in comparison to peptide bond formation.

In recent theoretical studies of peptidyl-tRNA hydrolysis, concerted and neutral stepwise mechanisms were studied by a small cluster model.\textsuperscript{13,14} The concerted six- and eight-membered transition states (Figure 2A, B) are similar to those in peptide bond formation,\textsuperscript{6} except that the nucleophile in this case is a water molecule. As mentioned above, the pH-rate profile and KSIE are distinctly different in peptidyl-tRNA hydrolysis and peptide bond formation. Therefore, a concerted TS does not seem to explain the underlying mechanism of the hydrolysis reaction. The proposed neutral stepwise mechanism (Figure 2C) is also similar to previously suggested transition states for peptide bond formation in which the carbonyl oxygen of the P-site substrate is protonated by the P-site 2′-OH group.\textsuperscript{15,16} However, it should be noted that the highest resolution crystal structures of the PTC with nonreactive substrate analogues do not support such a proton transfer due to an unfavorable geometry.\textsuperscript{17–19} The key steps in the neutral mechanism\textsuperscript{14} are the formation and collapse of a neutral tetrahedral intermediate, which both occur through a concerted proton transfer (Figure 2C). Hence, similar to the concerted transition states, the neutral stepwise mechanism is also not consistent with the pH–rate profile of peptidyl-tRNA hydrolysis. Furthermore, the calculated activation energies for all studied mechanisms were too high in comparison to the experimental value\textsuperscript{9,15} and the model used in these studies did not include any parts of the tRNA in the PTC.\textsuperscript{15,14}

The main goal of this work is to put forward a mechanism for peptidyl-tRNA hydrolysis that is compatible with the experimental observations: namely the pH dependence, KSIE, and activation energy. To achieve this goal, we constructed a large cluster model (~220 atoms) of the PTC which was used to study possible mechanisms by the density functional theory approach. To be able to compare our proposed mechanism to earlier suggestions, we also reexamined the previously proposed mechanisms by our cluster model. For each mechanism the KSIE was calculated for the rate-limiting step, which together with the calculated activation energies can be compared directly to the available experimental data. The results of our calculations suggest that the P-site A⁷⁶ O₂⁻ releases a proton to the bulk and acts as a general base in activating the nucleophilic water. The nucleophilic attack of water with simultaneous proton transfer to the deprotonated A⁷⁶ O₂⁻ is the only mechanism that is compatible with the measured activation energy and the magnitude of the KSIE. Moreover, the source of the pH dependence of translation termination seems to be the deprotonation of the 2′-OH group of the P-site tRNA rather than water.

\section*{Computational Methods}

The peptidyl transferase center was represented by a cluster model based on the most recent crystal structure of RF2 bound to 70S ribosome\textsuperscript{20} (PDB code 4V5J). The substrate analogue is

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Schematic representation of previously proposed mechanisms for peptidyl-tRNA ester hydrolysis. In the concerted six- (A) and eight-membered (B) transition states the proton transfer occurs simultaneously with nucleophilic attack of water and ester bond fission. The neutral stepwise mechanism (C) proceeds via a neutral tetrahedral intermediate, which is formed by nucleophilic attack of a water molecule on the carbonyl carbon and concerted proton transfer to carbonyl oxygen. Collapse of the intermediate yields the product, and in this step one proton is transferred from the carbonyl oxygen to A⁷⁶ O₃⁻ in a concerted eight-membered TS.}
\end{figure}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
\textbf{Mechanism} & \textbf{Activation Energy (kcal/mol)} \\
\hline
Concerted six-membered & 48.0 \\
Concerted eight-membered & 67.0 \\
Neutral stepwise & 84.0 \\
\hline
\end{tabular}
\caption{Comparison of activation energies for different mechanisms.}
\end{table}
(Phe-NH-A76) was transformed to a natural substrate (Ala-O-A76), and the terminal amine group of the substrate was acylated to mimic the next peptide bond. The nucleotides C2063, A2451, U2506, and U2585 from the 50S subunit and the conserved methylated Gln240 residue from RF2 were included in the model. The O5′ atoms of the nucleotides were capped with a methyl group to avoid artificial hydrogen bonds. Six water molecules were included in the model on the basis of similar crystal structures of the PTC and previous empirical valence bond (EVB) calculations. To account for the steric effect of surrounding parts of the ribosome, some atoms were kept fixed at their crystallographic position during geometry optimizations (Figure 3). The final model after addition of hydrogen atoms is composed of 224 atoms.

DFT calculations were performed with the Gaussian 09 package. Stationary points were optimized with the M06-2X functional and 6-31G(d,p) basis set. The electronic energies were then obtained by single-point calculations using M06-2X and the 6-311+G(2d,2p) basis set. Zero-point energies (ZPE) were obtained by single-point calculations using M06-2X and 6-31G(d,p) basis set which are corrected for ZPE and solvation effects. The resolution of the crystal structure (3.1 Å) is inadequate to provide reliable information about the positions of water molecules in the PTC. To acquire an accurate description of the PTC, six water molecules (W1–W6 in Figure 3) were therefore added to the model by comparison of the RF2-ribosome complex (PDB code 4V5J) with a higher resolution structure of the 50S subunit with substrate analogues and previous EVB simulations of peptidyl-tRNA hydrolysis. Waters W1–W3 were thus adopted from the crystal structures, while W4 and W5 were taken from the earlier molecular dynamics (MD) simulations and W6 was added to shield the active site from the continuum model. During the geometry optimizations it turns out that the last water molecule, in fact, occupies the same position as A75 O2′ in the P-site. Overall, this model consists of 224 atoms with a net charge of 0.

At the periphery of the system some atoms were kept fixed in order to maintain the geometry imposed by the surrounding part of the structure (Figure 3). During initial geometry optimizations, the substrate (acylated alanine ester) rotated from the initial position by ~60° around the ester bond in comparison to the X-ray crystal structure (Figure S1 in the Supporting Information). The orientation of the substrate analogue in this crystal structure is, however, most likely a suboptimal model for hydrolysis reaction. That is, not only is the electron density weak for the peptide part (Figure S2 in the Supporting Information) but also the substrate analogue has a (possibly positively charged) terminal amine group which would normally be far away from the PTC with a longer

RESULTS AND DISCUSSION

Validity of the PTC Model. The peptidyl transferase center was represented by a cluster model based on the crystal structure of RF2 bound to the 70S ribosome (PDB code 4V5J). The rRNA bases C2063 and A2451 were included in the model, since they participate in hydrogen bonds with crystallographically observed water molecules and the P-site A76 2′-OH group (Figure 3). Although the rRNA residues U2506 and U2585 do not interact directly with the reactive fragments, they make up the first shell of the PTC and possibly dictate the conformation of the nascent peptide chain.
substrate. In addition, the ester oxygen is replaced by an NH group, as in several other crystallized analogues, which changes its polarity. On the other hand, the orientation in our model very closely matches the most relevant crystal structure with bound P-site substrate in the peptidyl transfer reaction (PDB code 1VQN), which also contains a longer peptide chain (Figure S1B). In this conformation, the carbonyl oxygen of the substrate forms a hydrogen bond with the backbone amide of the conserved Gln240 residue (Figure 3). Since translation termination involves a full-length peptide chain, this orientation of the substrate appears to be a more reasonable representation for peptidyl-tRNA hydrolysis in comparison to that observed in the crystal structure of RF2 bound to the 70S ribosome (Figure S1A).

Different Possible Hydrolysis Mechanisms. Any proposed mechanism for peptidyl-tRNA hydrolysis on the ribosome must be compatible both with the experimental activation free energy of 18 kcal mol⁻¹ and the observed pH dependence of this reaction. In the concerted six- and eight-membered transition states, however, the nucleophilic attack of the water molecule occurs simultaneously with proton transfer to the P-site O₃' and ester bond fission (Figure 2A,B). Hence these TSs do not explain the pH dependence of peptidyl-tRNA hydrolysis. Nevertheless, we optimized these transition states (Figure 4) to compare various possible mechanisms within the same computational model. The activation energies for the six- and eight-membered TSs are 41.1 and 33.0 kcal mol⁻¹, respectively (Table 1). The lower activation energy of the eight-membered TS is due to participation of the extra water (W2 in Figure 4B) in the proton relay, which relieves enthalpic strain in the transition state. More favorable dipole interactions may also contribute.

The same effect was also observed in previous studies of peptide bond formation and peptidyl-tRNA hydrolysis. In the eight-membered TS, the O₂' atoms of C2063 and A2451 form hydrogen bonds with W2 and A76 O₂' (Figure 4B). These interactions are, however, lost in the six-membered TS (Figure 4A) and A2451 O₂' instead interacts with the attacking water (W1). In both transition states the developing charge on the carbonyl oxygen of the substrate (O in Figure 4) is stabilized by hydrogen bonding to W4 and the backbone amide of the conserved Gln240. The side chain of Gln240 has similar conformations in two different TSs, which are consistent with earlier crystal structures and the predictions from MD simulations. Most importantly, the carbonyl oxygen of the Gln240 side chain hydrogen bonds to the nucleophilic water molecule (Figure 4), thereby orienting it for attack on the substrate as predicted earlier.

The proposed neutral stepwise mechanism proceeds through formation of a neutral tetrahedral intermediate (Figure 2C). The intermediate (IM) is formed by nucleophilic attack of the catalytic water molecule on the substrate carbonyl carbon...
Figure 5. Transition states of the neutral stepwise mechanism. Formation of the tetrahedral intermediate occurs via a six-membered transition state (TS1) in which the carbonyl oxygen (O) is protonated. The ester bond breaks with simultaneous proton transfer from the attacking water to O3′ in an eight-membered transition state (TS2). Only residues in the first solvation shell are shown, and nonpolar hydrogen atoms are omitted for clarity.

Figure 6. Schematic representation of the base-catalyzed mechanism. Following the initial deprotonation of the A76 2′-OH group, the tetrahedral intermediate (IM) is formed by nucleophilic attack of a water molecule with simultaneous proton transfer to A76 O2′ (TS1). Ester bond fission occurs in the second transition state (TS2), which yields the protonated product.

Figure 7. Transition states for the base-catalyzed mechanism. The optimized structures of the TSs for tetrahedral intermediate formation (TS1) and decomposition (TS2) are shown. The side chain of Gln240 interacts with the attacking water in TS1 and with the leaving peptide amide in TS2. In both transition states, the Gln240 backbone amide and W4 stabilize the negative charge of carbonyl oxygen (O). Only residues in the first solvation shell are shown, and nonpolar hydrogen atoms are omitted for clarity.
with simultaneous proton transfer from the attacking water to the carbonyl oxygen (TS1 in Figure 5). This proton transfer is mediated by the P-site A76 2′-OH group, which results in a six-membered TS with an activation energy of 23.4 kcal mol\(^{-1}\). However, the proton transfer from O2′ to the substrate carbonyl oxygen requires a significant rotation of the substrate, which is not seen in any crystal structures, and there is no interaction between the Glu240 side chain and the reacting groups. In the second step, the C–O ester bond breaks and one proton is transferred simultaneously from IM to the P-site A76 O3′ (Figure 2C), where it was suggested that the proton transfer would again be mediated by A76 O2′ and a water molecule. However, the predicted activation energy barrier\(^{14}\) for this step was over 37 kcal mol\(^{-1}\). In our PTC model, we observed that the 2′-OH group of C2063 is better positioned to relay the proton from IM to the P-site A76 O3′. Since the C2063 2′-OH is chemically equivalent to the P-site A76 2′-OH, we optimized a transition state in which the proton transfer is mediated by the former group and an auxiliary water (TS2 in Figure 5). The activation energy for this transition state is 30.4 kcal mol\(^{-1}\), which is approximately 8 kcal mol\(^{-1}\) lower than the alternative neutral mechanism.\(^{14}\) Overall, the activation energy of this mechanism is very similar to that obtained from the concerted eight-membered TS (Table 1), indicating that initial protonation of substrate carbonyl oxygen does not yield any notable catalytic effect. It can further be noted that there is no significant entropy contribution observed for this reaction experimentally that could bring the free energy barrier down to the observed value.\(^{9}\)

**Base-Catalyzed Mechanism.** The pH dependence of peptidyl-tRNA hydrolysis indicates that an ionizable group with \(pK_a > 9\) participates in the reaction.\(^{9,11}\) This suggested that hydroxide ion could in fact act as the nucleophile in the hydrolysis reaction.\(^{9–11}\) To investigate this, we replaced the attacking nucleophilic water with a hydroxide ion. In the geometry optimization, however, the hydroxide ion abstracts one proton from the A76 2′-OH group. This proton transfer is consistent with a lower \(pK_a\) of A76 2′-OH in comparison to water, as was observed for the relevant model compound 3′-O-methyladenosine with \(pK_a = 13.70.\)\(^{12}\) Hence the deprotonated A76 O2′ state was taken as the reference state (state R′ in Figure 6) for the base-catalyzed hydrolysis. In this state, the negative charge on A76 O2′ is stabilized by hydrogen bonding to the A2451 2′-OH group, W1, and the backbone amide of Gln240.

A negatively charged tetrahedral intermediate is then formed by the nucleophilic attack of W1 on the carbonyl group of the peptidyl-tRNA with simultaneous proton transfer to A76 O2′ (Figure 6). The activation energy for this step (TS1) is found to be 7.4 kcal mol\(^{-1}\) relative to R′ (Figure 7). The negative charge of the tetrahedral intermediate is stabilized by hydrogen bonding to the backbone amide of Gln240 and W4, as predicted earlier.\(^{1,3,10}\) This brings the relative energy of the intermediate down to \(-1.5\) kcal mol\(^{-1}\) in comparison to R′. Subsequently, fission of the bond between A76 O3′ and the substrate carbon occurs almost spontaneously with only a minor 2.6 kcal mol\(^{-1}\) barrier (TS2 in Figure 7), thereby forming the protonated product (Figure 6). The activation and reaction energy for this step are thus \(\Delta E^\ddagger = 1.0\) kcal mol\(^{-1}\) and \(\Delta E^e = -3.1\) kcal mol\(^{-1}\) relative to the state R′, respectively. Proton transfer from the protonated product to the leaving A76 O3′ (Figure 6) has a very low barrier and yields the final product, with a reaction energy of \(-18.0\) kcal mol\(^{-1}\) relative to R′.

However, to obtain the overall reaction energy of the base-catalyzed mechanism, the initial proton transfer from the A76 2′-OH group to bulk solvent (at pH 7.5 as in the experiments) must be taken into account. Such a calculation is, however, very difficult to carry out by any computational approach, since it is equivalent to evaluating the absolute energy of a (negative) charge in the complex ribosome environment, with numerous phosphate charges and counterions.\(^{10}\) Therefore, the most reasonable and unbiased estimate of the energy cost for this step is to assume that the 2′-OH p\(K_a\) value is unperturbed by the ribosome environment, as the crystal structures of the PTC give no reason to expect any unusually large p\(K_a\) shifts. This is also supported by the small p\(K_a\) shifts observed experimentally for the attacking amine in the peptidyl transfer reaction.\(^{7}\)

Hence, the reaction free energy can then be estimated to be the same as in aqueous solution and is given by \(\Delta G^0 = 1.36(pK_a - \text{pH})\). This gives a free energy cost for the initial proton transfer step of 8.4 kcal mol\(^{-1}\) using the estimated p\(K_a\) of 13.7 for the A76 2′-OH group\(^{12}\) and a solution pH of 7.5 at 25 °C. Although it could perhaps be argued that the PTC environment would not stabilize the A76 O2′ anion as much as the solution phase, it should be noted that the PTC is highly preorganized by hydrogen bonding to the relevant groups, which reduces the entropy cost of solvent reorganization that accompanies the water reaction.\(^{30,31}\) Therefore, the free energy estimate of the initial deprotonation of A76 O2′ appears as a very reasonable approximation. Hence, for the base-catalyzed mechanism the rate-limiting nucleophilic attack of water on the substrate carbonyl carbon (TS1) is predicted to have an overall activation energy of 15.8 kcal mol\(^{-1}\) relative to the state R (Figure 8). Considering that the experimentally determined entropy\(^9\) contribution is \(-T\Delta S^\ddagger = 1.6\) kcal mol\(^{-1}\), this would yield a predicted free energy barrier of 17.4 kcal mol\(^{-1}\), which is in excellent agreement with the experimental data.\(^9,11,26\)

In this mechanism, the deprotonated P-site A76 O2′ thus acts as the general base that activates the nucleophilic water. The ionization of A76 O2′ is therefore the most probable reason for the pH dependence of the hydrolysis reaction. This is also in agreement with mutation experiments of A76 O2′ where elimination of the 2′-hydroxyl decreased the reaction rate by \((\sim 7 \times 10^{-5})\)-fold \((\sim 6\) kcal mol\(^{-1}\)) in the RF1-catalyzed reaction.\(^{10}\) This decrease in the reaction rate would thus partially be due to the change of the general base from the P-site A76 O2′ (p\(K_a = 13.70\)) to water (p\(K_a = 15.74\)), which accounts for an \(~(1 \times 10^{-5})\)-fold \((\sim 3\) kcal mol\(^{-1}\)) decrease in the reaction rate. A similar effect was also, in fact, observed for
the uncatalyzed ribosome reaction in the absence of release factor\textsuperscript{10} and on comparison of the hydrolysis of Leu-tRNA and Leu-ethyl ester in aqueous solution.\textsuperscript{12}

**Kinetic Solvent Isotope Effect.** To further investigate the consistency of the proposed mechanisms with experimental observations, the KSIEs were calculated by replacing polar hydrogen atoms with deuterium (Table 1). In these calculations, only the ZPE contribution was taken into account, which has been shown to yield reliable results.\textsuperscript{33} For the concerted six- and eight-membered transition states, we obtained ZPE differences of 0.52 and 1.14 kcal mol\textsuperscript{-1} between the TSs and R which gives KSIEs of 2.4 and 6.8 for the six- and eight-membered TSs, respectively. In the neutral stepwise mechanism, the ZPE difference for TS2 is 0.58 kcal mol\textsuperscript{-1}, which yields a KSIE of 2.7 for this transition state. For the rate-limiting step (TS1) of the base-catalyzed mechanism, the ZPE difference was found to be 0.46 kcal mol\textsuperscript{-1}, which yields a KSIE of 2.16 relative to the state R'. The equilibrium isotope effect (EIE) for the initial deprotonation step was estimated from the 2-chloroethanol dissociation equilibrium in water and deuterium oxide. That is, proton transfer from CICH\textsubscript{2}OH (pK\textsubscript{a} = 14.31)\textsuperscript{34} to bulk water at pH 7 yields a reaction free energy of 10.0 kcal mol\textsuperscript{-1}. For deuterated CICH\textsubscript{2}OD (pK\textsubscript{a} = 14.31)\textsuperscript{35} at neutral pH 7.48 the corresponding reaction free energy is 10.2 kcal mol\textsuperscript{-1}, which yields an EIE of 1.6. Using this value as an approximation for the EIE of initial substrate deprotonation on the ribosome, the overall isotope effect for the rate-limiting step in the base-catalyzed mechanism is found to be 3.4 (Table 1). Since the pK\textsubscript{a} of 2-chloroethanol is very similar to that of the P-site A76 2'-OH group,\textsuperscript{12} the calculated EIE for initial deprotonation is a reasonable approximation.

The calculated KSIE of 3.4 for the base-catalyzed mechanism is thus also in good agreement with the measured value for the RF2-catalyzed reaction\textsuperscript{9} of 4.1, and it lies between the values for the six- and eight-membered concerted TSs. Furthermore, it is important to emphasize that the base-catalyzed mechanism is that yielding the lowest activation energy and this would be true even if the A76 2'-OH group had a pK\textsubscript{a} as high as that of water, in which case the activation energy would increase by 3 kcal mol\textsuperscript{-1}. Importantly, it is also the only mechanism consistent with the observed pH dependence of the peptide release reaction. Hence, these results strongly support the base-catalyzed mechanism in the peptidyl-tRNA hydrolysis reaction.

**CONCLUSION**

The peptidyl-tRNA hydrolysis reaction is one of the two processes catalyzed by the ribosomal peptidyl transferase center, and it is required for release of the newly synthesized polypeptide in termination of protein synthesis. Despite the importance of this reaction and a wealth of experimental results, the exact chemical mechanism has remained puzzling. In contrast to peptide bond formation, the peptide release rate is dependent on hydroxide ion concentration in the RF1- and RF2-catalyzed reactions.\textsuperscript{5,11} This suggests that ionization of either the nucleophilic water or P-site A76 2'-OH group is required for efficient peptidyl-tRNA hydrolysis. Among the various possible reaction schemes, our calculations now show that the mechanism in which ribosome "waits" for spontaneous ionization of the P-site A76 2'-OH group is that which gives the closest agreement with the experimentally derived activation free energy. Importantly, it is also the only proposed mechanism that can account for the observed pH dependence of the reaction. We further note that this conclusion does not really depend on our assumption of an unperturbed 2'-OH pK\textsubscript{a}, since the base-catalyzed mechanism is predicted to be favored by as much as 11 pK\textsubscript{a} units.

Interestingly, MD/EVB simulations of this type of mechanism could also explain the effects of RF1 mutations on the termination reaction with a P-site dA76 substrate, which shows a similar pH dependence.\textsuperscript{10} Here, it may also be noted that earlier MD/EVB simulations of the termination reaction gave a large catalytic effect on the ribosome also for a neutral mechanism.\textsuperscript{1,5,10} This is perhaps not so strange, considering that the EVB transition state structures for neutral and anionic mechanisms were very similar, apart from the removal of one proton from the system.\textsuperscript{10} That is, the stabilizing effect from the Gln240 side chain and backbone, as well as the key water molecule, W4, is conserved for mechanisms that do not involve protonation of the substrate carbonyl (Figures 4 and 7). The absolute EVB free energy barrier is, however, dependent on the calibration against the uncatalyzed solution reaction, for which there are no unambiguous data regarding the difference in activation barriers between hydroxide and neutral water attack mechanisms. On the basis of a study of the spontaneous hydrolysis of Leu-tRNA and Leu-ethyl esters in aqueous solution, it was suggested that the mechanism is indeed dominated by hydroxide ion attack.\textsuperscript{32} However, in this case the leucine amine is protonated, which could facilitate nucleophilic attack, and the postulated mechanism is kinetically indistinguishable from water attack on the neutral ester. Nevertheless, in view of the recently reported linear pH dependence\textsuperscript{7} also of the uncatalyzed hydrolysis of fMet-tRNA\textsubscript{Met} (with no charged amine) it is likely that the spontaneous hydrolysis of a peptidyl-tRNA ester would also follow the substrate-assisted mechanism suggested herein.

Hence, in this mechanism the deprotonated A76 2'-OH group acts as a general base that activates the attacking water. In the rate-limiting step (TS1) the nucleophilic attack on the substrate carbonyl carbon occurs simultaneously with proton transfer from water to the P-site A76 O2'. This single proton transfer is also consistent with the measured KSIE of 4.1 and linear proton inventory plot.\textsuperscript{9} The proposed mechanism further supports the role of the Gln240 in stabilizing the partially negative substrate carbonyl oxygen and in orienting the nucleophile for efficient attack on the ester carbon atom.\textsuperscript{1} Overall, this substrate-assisted base catalysis pathway appears to be consistent with all available experimental data. The question then naturally arises why such a mechanism does not seem to be operational in peptide bond formation, where the reaction does not show the same linear pH dependence as in peptide release.\textsuperscript{7,35} The reason for this could be that one proton has already been removed from the system, when the attacking A-site amine becomes deprotonated, and no more protons can be lost if an ionized leaving group is to be prevented. In the hydrolysis reaction, on the other hand, the O3' leaving group can still be protonated by the carboxylic acid moiety of the leaving peptide, which is a downhill process.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.6b02842.

Comparison of the orientation of the substrate in crystal structures with different optimized transition states and the coordinates of all optimized states (PDF)
REFERENCES

DFT, density functional theory; RF, release factor; PTC, peptidyl transferase center; KSIE, kinetic solvent isotope effect; TS, transition state

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ABBREVIATIONS

DFT, density functional theory; RF, release factor; PTC, peptidyl transferase center; KSIE, kinetic solvent isotope effect; TS, transition state

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Notes

The authors declare no competing financial interest.

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