An Uncharged Amine in the Transition State of the Ribosomal Peptidyl Transfer Reaction

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SUMMARY

The ribosome has an active site comprised of RNA that catalyzes peptide bond formation. To understand how RNA promotes this reaction requires a detailed understanding of the chemical transition state. Here, we report the Brønsted coefficient of the α-amino nucleophile (β_{nuc}) using a series of puromycin derivatives. Both 50S subunit and 70S ribosome catalyzed reactions displayed linear free-energy relationships with slopes close to zero under conditions where chemistry is rate limiting. These results indicate that at the transition state the nucleophile is neutral in the ribosome catalyzed reaction, in contrast to the substantial positive charge reported for typical uncatalyzed aminolysis reactions. This suggests that the ribosomal transition state involves deprotonation to a degree commensurate with nitrogen-carbon bond formation. Such a transition state is significantly different from that of uncatalyzed aminolysis reactions in solution.

INTRODUCTION

The ribosome is the macromolecular machine that catalyzes cellular protein synthesis. The ribosome promotes amide bond formation within the peptidyl transferase center (PTC) of the 50S ribosomal subunit. Crystallographic studies have established that RNA surrounds the PTC, demonstrating that the ribosome is a ribozyme(Ban, Nissen et al., 2000). The two ribosomal substrates, the peptidyl-tRNA and the aminoacyl-tRNA bind to two adjacent sites within the PTC (P site and A site, respectively). In peptidyl transfer (PT), the α-amino group of the A-site aminoacyl-tRNA attacks the ester of the P-site peptidyl-tRNA. This results in the deacylation of the P-site tRNA and a peptide chain that is extended by one amino acid.

The ribosome enhances the PT reaction approximately 10^7-fold over the rate of comparable reactions in solution(Rodnina, Beringer et al., 2006). Several mechanisms have been proposed to account for this observed rate enhancement including general acid-base catalysis(Muth, Ortoleva-Donnelly et al., 2000; Nissen, Hansen et al., 2000), catalysis by substrate alignment (Sievers, Beringer et al., 2004) and substrate-assisted catalysis(Dorner, Panuschka et al., 2003; Weinger, Parnell et al., 2004; Trobro and Aqvist, 2005). Biochemical, mutational, and recent structural evidence suggest that the general acid-base mechanism as originally proposed

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is unlikely. The P-site A76 2′-OH is situated close to the attacking α-amine and O3′ leaving group, making it one of the few functional groups in a position to be directly involved in catalysis(Schmeing, Huang et al., 2005; Schmeing, Huang et al., 2005). Removing the A76 2′-OH decreases the rate of peptide bond formation by at least one million-fold(Weinger, Parnell et al., 2004). The catalytic importance and physical proximity of this key functional group has led to proposals that it is involved in a proton shuttle between the α-amine and the O3′ leaving group(Das, Bhattacharyya et al., 1999; Schmeing, Huang et al., 2005; Rodnina, Beringer et al., 2007).

A detailed knowledge of the transition state of the PT reaction will aid in understanding the catalytic mechanism of the ribosome. One method for defining the transition state is Brønsted analysis. The Brønsted plot is a linear free energy relationship that describes the correlation between the log of the reaction rate and the pK_as of the respective nucleophile or leaving group. By using a series of modified substrates with different pK_as, the magnitude and sign of the Brønsted coefficient (β) can be determined from the slope of the Brønsted plot. The Brønsted coefficient reflects the change in charge of an ionizing group between the ground state and the transition state. It is also indicative of the changes in bonding that occur in the transition state (Jencks, 1987; Fersht, 1999).

Except for highly activated nucleophiles or leaving groups, Brønsted coefficients for the nucleophile (β_{nuc}) values in solution are typically between 0.8-0.9 for primary and secondary amines(Bruice and Lapinski, 1958; Jencks and Carriuolo, 1960; Jencks and Gilchrist, 1968; Satterthwait and Jencks, 1974). Positive values close to one suggest that the α-amine has a significant positive charge in the transition state, retaining the α-amine protons as the nitrogen-carbon (N-C) bond forms. Jencks proposed a stepwise reaction mechanism involving two tetrahedral intermediates (T± and T−). The α-amine in the first tetrahedral intermediate (T±) retains both protons and is deprotonated via a proton transfer to form the second intermediate (T−). This proton transfer limits the rate and a β_{nuc} close to 1 indicates that the transition state closely resembles the T± intermediate(Satterthwait and Jencks, 1974).

Does PT on the ribosome go through the same transition state as uncatalyzed aminolysis in solution? Determining the Brønsted coefficient of the α-amino group of the A-site substrate would begin to address this question and help to define the nature of the catalytic contribution provided by the ribosome. A ribosomal Brønsted coefficient of ~0.8 would suggest that the transition state of both the catalyzed and uncatalyzed reactions are the same. A value that deviates significantly would indicate that the ribosome proceeds through an alternative transition state. Previously, we reported that β, β-difluorophenylalanyl- ($pK_a < 5.0$) reacted 90-fold faster than the phenylalanyl derivative at acidic pH. At higher pH's the rates of the phenylalanyl and fluorinated phenylalanyl deriviatives varied only 2-fold, suggesting an unexpectedly small β_{nuc} value. However, the limited sample size and the use of only the 50S fragment assay were insufficient for mechanistic conclusions(Okuda, Seila et al., 2005). Here we apply Brønsted analysis to an expanded series of derivatives and establish that the transition states for the PT reaction catalyzed by both the 50S subunit and 70S ribosome substantially differ from that of the uncatalyzed reaction.

RESULTS

We prepared a series of puromycin derivatives (A-site substrates) (Figure 1) and used these compounds to determine the Brønsted coefficient for the nucleophilic α-amine. The substrates were designed to be structurally similar to the parental substrate with minor modifications to perturb the pK_a of the nucleophilic amino group. A valid enzymatic Brønsted analysis requires that the modifications do not alter the binding of the substrate to the enzyme or the isostericity of the substrates, which significantly limits the diversity of modifications that can be made.

We focused on the amino acid phenylalanine and its derivatives with either β-carbon or aromatic ring substitutions. The pK_a of the α-amine was perturbed by introducing conservative electron withdrawing and donating substituents into the phenylalanine side chain. The result was a series of substrates whose pK_as ranged from <5 to 7.2 (Figure 1).

$β_{nuc}$ Measurements on the 50S Ribosomal Subunit

The aminolysis rate for each puromycin (Pmn) derivative was determined under single turnover conditions using the modified 50S fragment assay(Schmeing, Seila et al., 2002; Okuda, Seila et al., 2005). The radiolabled P-site substrate, CCApcb (phenylalanine-caproic acid-biotin) was present in trace amounts in the presence of a saturating concentration of 50S subunits and A-site substrate(Seila, Okuda et al., 2005). The reactions were performed at pH 8.5, to ensure that all substrates were in the amino rather than the unreactive ammonium form (Supplemental Figure 1, Table 1). Eight substrates reacted efficiently [CPmn(β, β-F-Phe), CPmn(3, 4-Cl-Phe), CPmn(2-F-Phe), CPmn(3, 4-F-Phe), CPmn, CPmn(Phe), CPmn(4-CH$_3$-Phe), and CPmn (4NH$_2$-Phe)] while four of the substrates [CPmn(2, 4-Cl-Phe), CPmn(2-NO$_2$-Phe), CPmn(2-CF$_3$-Phe) and CPmn(F$_5$-Phe)] were inactive. Three of the inactive and none of the active derivatives contained a bulky substitution at the ortho position of the benzyl ring. The inhibition constant for CPmn(F$_5$-Phe) was comparable to the K_M for CPmn (data not shown), suggesting that the derivative binds but is unreactive. Such steric effects are independent of the derivative pK_a and preclude the use of these substrates in a Brønsted analysis.

The rate of the PT reaction on 50S subunits was measured for each of the eight active compounds at substrate concentrations 3 to 4-fold above the K_M of CPmn. Raising the concentrations 8-fold above the K_M value did not change the rate, which implies that the substrate concentration was saturating and therefore the observed rates correspond to the chemical step, k_{pep}. The PT rates for all A-site substrates tested varied less than 2.5-fold, ranging from 0.48 min^{-1} to 1.1 min^{-1}. The $β_{nuc}$ for peptide bond formation on 50S subunits was determined from the slope of log k_{pep} versus the pK_a of the α-amino group of each aminoacyl substrate, resulting in a Brønsted coefficient of 0.06 (Figure 2A). The observed linearity for the plot indicates no change in the rate-determining step(Jencks, 1987).

$β_{nuc}$ Measurements on the 70S Ribosome

Brønsted analysis was performed with the 70S initiation complex using a single-turnover kinetic assay(Katunin, Muth et al., 2002; Beringer, Bruell et al., 2005). f[^{3}H]Met-tRNA^{fMet} was preloaded into the P-site to form the 70S initiation complexes and the rate of dipeptide formation at pH 8.5 was measured for Pmn and its derivatives. Only four derivatives [Pmn (3,4-Cl-Phe), Pmn(3,4-F-Phe), Pmn(4-CH$_3$-Phe), and Pmn(4-NH$_2$-Phe)] were soluble at the concentrations required to reach substrate saturation. Previous reports found that 20% DMSO had no effect on the Pmn reaction rate(Katunin, Muth et al., 2002). We found that up to 60% DMSO was tolerated with the only consequence being a slight increase in the binding constant (K_M) for Pmn (Supplemental Figures 2 and 3).

The rates of peptidyl transfer by the 70S ribosome for all of the puromycin derivatives were very similar. Data were collected for Pmn(β, β-F-Phe), Pmn(3, 4-Cl-Phe), Pmn(3, 4-F-Phe), Pmn, Pmn(Phe), and Pmn(4-CH$_3$-Phe) in the presence of DMSO (Table 2). Although the pK_a values of these derivatives range from <5 to 7.2, the rate of aminolysis by these six substrates varied less than 2-fold, from 13.2 s^{-1} to 22.4 s^{-1}. Values for Pmn(β-F-Phe) and Pmn (4-NH$_2$-Phe) are not obtained because an upper limit to solubility was reached before saturation occurred.

A subset of analogs was sufficiently soluble in buffer B in the absence of DMSO to reach saturating concentrations. These substrates covered the full range of pK_as in the collection. For

the Pmn derivatives Pmn(β,β-F-Phe), Pmn(β-F-Phe), Pmn, and Pmn(Phe) (Table 2) all rates were no more than three fold different, ranging from 12 s^{-1} to 37 s^{-1}.

The rate of PT reaction on 70S ribosomes, like that of the 50S subunits, is independent of the basicity of the nucleophile (Figure 2B & 2C). The Brønsted coefficients for the PT reaction on the 70S ribosomes were −0.06 and −0.27 in the presence and absence of DMSO, respectively. The linearity of the plot over the entire pK$_a$ range signifies that there is no change in the rate-determining step for the different analogues(Jencks, 1987).

DISCUSSION

The Brønsted coefficient of the nucleophilic α-amine (β$_{nuc}$) in the PT reaction on the ribosome was determined for reactions catalyzed by both the 50S subunit and 70S ribosome. A series of puromycin-like substrates were synthesized with conservative, nearly isosteric substitutions of the phenylalanine side chain that resulted in pK$_a$s for the α-amino group ranging from <5 to 7.2. Using both the modified 50S fragment reaction and the 70S initiation complex reaction, the β$_{nuc}$ was found to be approximately zero, suggesting that the rate of peptidyl transfer is independent of the pK$_a$ of the nucleophilic amine. Since the magnitude and sign of the β$_{nuc}$ value reflects the change in charge on the attacking nucleophile between the ground state and the transition state, these near zero β$_{nuc}$ values suggest that the amine remains neutral in the PT transition state. Comparable β$_{nuc}$ values for reactions on the 50S subunit and the 70S ribosome support the similar intrinsic PT activity of the 50S subunits and 70S ribosomes (Wohlgemuth, Beringer et al., 2006).

In order to properly interpret the measured Brønsted coefficients, it is important to ensure that the assays are measuring the chemical rate and not another step in the pathway. If chemistry is not rate limiting, a near zero β$_{nuc}$ may arise because amine basicity would not affect the non-chemical steps of the assay. Several independent lines of evidence indicate that amide bond formation is rate limiting for both assays. In the 50S modified fragment assay: (i) there is a rapid-equilibrium between reactants and the bound complex (no forward commitment to catalysis), indicating that there is no rate limiting step prior to chemistry(Seila, Okuda et al., 2005); (ii) kinetic isotope effect (KIE) measured for the A-site substrate indicated that bond formation is at least partially rate limiting(Seila, Okuda et al., 2005); and (iii) a less-reactive nucleophile, hydroxypuromycin (the α-amino group is replaced with a hydroxyl) decreased the peptidyl transfer rate at least 20-fold(Seila, Okuda et al., 2005). For the 70S assay: (i) puromycin binding to the A site is not rate-limiting because peptidyl-tRNA substrates with different peptide lengths react at different rates. For example there is a 56-fold difference in the reaction rate of puromycin with fMet-tRNA^{fMet} versus fMetAlaAsnMetPheAla-tRNA^{Ala}(Katunin, Muth et al., 2002). (ii) Substitution of puromycin with hydroxypuromycin decreases the rate of peptidyl transfer 200-fold(Katunin, Muth et al., 2002). (iii) Pmn binding into the PT center is not rate-limiting(Katunin, Muth et al., 2002; Sievers, Beringer et al., 2004). These observations argue that the near zero β$_{nuc}$ value does not result from monitoring a non-chemical step in the reaction pathway.

The β$_{nuc}$ values measured for the PT reaction on 50S subunits and 70S ribosomes differ significantly from those reported for most uncatalyzed aminolysis reactions. A β$_{nuc}$ near zero for peptide bond formation by the ribosome is inconsistent with formation of positive charge on the α-amine in the transition state. If this occurred, as in uncatalyzed aminolysis reactions (Bruice and Lapinski, 1958; Jencks and Carriuolo, 1960; Jencks and Gilchrist, 1968; Satterthwait and Jencks, 1974), then the addition of electron-donating groups to the amino acid side chain would stabilize the developing charge resulting in a positive correlation between reactivity and basicity(Zeeberg and Caplow, 1973). Also unlikely is the removal of the proton before nucleophilic attack, because the energetics associated with forming the amine anion are

highly unfavorable(Blackburn and Jencks, 1968). The observed Brønsted coefficients indicate that the nucleophile remains uncharged in the ribosomal transition state, which suggests that the degree of deprotonation of the amine is similar to the degree of N-C bond formation. A transition state with a neutral nucleophile has implications for mechanistic models of the ribosomal reaction. Although the β_{nuc} value does not fully define the transition state, it excludes several possibilities. Any mechanisms that require a transition state with significant positive charge development on the α-amine can be eliminated.

Several mechanisms have been proposed for the PT reaction. The first is equivalent to the solution aminolysis mechanism and proceeds stepwise through two intermediates (T± and T −)(Jencks and Gilchrist, 1968; Satterthwait and Jencks, 1974) (Figure 3). The second is also stepwise but involves only a single intermediate (T±) (Figure 4). This is one form of the "proton shuttle" mechanism and involves concerted proton transfer with C-O bond cleavage that resolves the T± intermediate directly to products(Das, Bhattacharyya et al., 1999; Schmeing, Huang et al., 2005; Trobro and Aqvist, 2005). The third mechanism is a fully concerted reaction without intermediates and is a second variant of the "proton shuttle" proposal (Figure 5). This mechanism requires that all bond formation, cleavage, and proton transfers occur simultaneously(Dorner, Polacek et al., 2002; Changalov, Ivanova et al., 2005). Each of these mechanisms can now be evaluated in light of the β_{nuc} value determined for the ribosomal reaction.

Assuming the mechanism with two intermediates(Jencks and Gilchrist, 1968; Satterthwait and Jencks, 1974), (Figure 3), there are three transitions that could be rate limiting and each can be considered with regard to the experimentally determined β_{nuc} value. If the rate-limiting step is nucleophilic attack and occurs upon the transition from substrates to the T± intermediate (Figure 3A), any degree of N-C bond formation without equivalent proton removal would increase the positive charge on the nucleophilic α-amine. The only transition state consistent with the observed β_{nuc} would be an early one with minimal N-C bond formation. Low β_{nuc} values of 0.1-0.2 were observed for model aminolysis reactions in solution for substrates with extreme pK_as, which fell in the non-linear regions of reported Brønsted plots. The non-linearity of these plots was interpreted to denote a change in the rate-limiting step. Jencks interpreted the near zero β_{nuc} values in these regions to indicate that nucleophilic attack was rate-limiting, resulting in an early transition state with little amide bond character and full retention of both amine protons(Jencks and Gilchrist, 1968; Satterthwait and Jencks, 1974).

A second possibility in this two-intermediate mechanism is that abstraction of the proton from T± to produce the T- intermediate is rate-limiting (Figure 3B). A near zero β_{nuc} requires that the transition state must involve significant deprotonation, resulting in a transition state similar to the T- intermediate. If the rate-limiting step occurs after the T- intermediate, then C-O bond breaking would take place in the transition state. In this case, the β_{nuc} value is not informative (Figure 3C) because inductive effects introduced by substitutions near the nucleophile are not conducted over the distance required to alter the leaving group. Thus, even assuming a stepwise reaction mechanism comparable to a typical aminolysis reaction in solution, the transition state is significantly different. Instead of resembling T±, the transition state either resembles substrates (Figure 3A), the T- intermediate (Figure 3B), or a transition state following the T-intermediate (Figure 3C).

The other mechanistic proposals that have been specifically suggested for the ribosome can also be evaluated in light of the Brønsted results. A stepwise mechanism with only one intermediate (T±) (Figure 4) has two transitions that could limit the overall rate(Das, Bhattacharyya et al., 1999;Schmeing, Huang et al., 2005;Trobro and Aqvist, 2005). This "proton shuttle" mechanism has been proposed based upon both *in silico*(Das, Bhattacharyya et al., 1999;Trobro and Aqvist, 2005) and crystallographic studies(Schmeing, Huang et al.,

2005). In this mechanism, the 2′-OH of the P-site A76 shuttles protons between the α-amine and the 3′-O at the same time as the C-O bond cleaves. If the rate-limiting step is nucleophilic attack toward T± formation, the β_{nuc} data are only consistent with a very early transition state resembling substrates for the same reasons as described above (Figure 4A). However, if resolution of the tetrahedral intermediate to products is rate-limiting (Figure 4B), then a zero β_{nuc} value would only be possible in a transition state with significant C-O bond cleavage coupled to complete α-amine deprotonation. In this mechanism, the β_{nuc} data constrain the transition state to one that is very late with a structure similar to products.

The fully concerted "proton shuttle" mechanism with no intermediates (Figure 5) (Dorner, Polacek et al., 2002;Changalov, Ivanova et al., 2005) requires that N-C bond formation, C-O bond cleavage, and proton-shuttling from the α-amine to the leaving group 3′-O all occur simultaneously. A near zero β_{nuc} value is expected throughout the entire reaction coordinate as N-C bond formation occurs concurrently with α-amine proton abstraction. This coupling of bond formation and proton abstraction removes any relationship between the β_{nuc} value and the extent of bond formation(Fersht, 1999). KIE and Brønsted analysis of the O3′ leaving group would be particularly useful for addressing the feasibility of this model.

A fourth model was proposed based upon a model system for the PT reaction. Changalov and Petkov argued that N-C bond formation is commensurate with α-amine deprotonation based on kinetic isotope effect (KIE) and Hammett correlation data for the transesterification of 2′/3′-O-p-substituted 2′-O-trityl adenosines and 2′-deoxyadenosines. Although these experiments were performed in solution, an organic base (DBU) was used as a catalyst and aprotic, apolar solvent conditions were chosen to mimic the interior of the ribosome. The KIEs indicate that a proton is "in flight" during the rate-determining transition state. The structure-activity relationship suggests that the rate-determining step for the model reaction is the formation of a tetrahedral intermediate that is similar to the T- intermediate, which they used as evidence for concerted nucleophilic attack and proton transfer(Changalov and Petkov, 2007). In this mechanism, the reaction proceeds from substrates to T- without forming the T± intermediate. This mechanism is also consistent with the β_{nuc} value for the ribosome, but it requires deprotonation of the 2′-OH by a strong base to activate the 2′-O for deprotonation of the amine. There are no strong candidates for such an activating group within the ribosomal active site and chemical modification of the P-site A76 2′-OH indicates that it remains neutral in the transition state (Huang et al., submitted). Although the precise transition state cannot be fully defined from this β_{nuc} value, the possible mechanisms that remain all differ significantly from the uncatalyzed reaction in solution.

There are examples of low β_{nuc} coefficients for enzymes that have been interpreted to represent concerted or partially concerted reaction mechanisms. Bender investigated the serine protease-catalyzed cleavage of amides (a reaction resembling the reverse reaction of peptide bond formation) and proposed that the reaction proceeds through an 'S_N2-like' rather than a step-wise mechanism because proton abstraction and nucleophilic attack are coupled(Komiyama and Bender, 1979). Inward and Jencks measured aminolysis rates on an acylenzyme, furoyl-chymotrypsin, and reported β_{nuc} values of 0.13 – 0.19. They concluded that unassisted nucleophilic attack by the free amine could not occur and the proton must be removed either before or during the transition state(Inward and Jencks, 1965). Finally, a β_{nuc} near zero was measured for amide bond synthesis by acetyltyrosylchymotrypsin. Deuterium isotope effects provided further support for the authors' conclusion that proton abstraction is coupled with amine attack(Zeeberg and Caplow, 1973). These examples provide evidence that enzymes can perform aminolysis through different pathways than observed in solution and may be illustrative of the ribosomal reaction mechanism.

SIGNIFICANCE

This study explores a basic mechanistic question: Does the ribosomal peptidyl transfer reaction proceed through the same transition state as observed for a typical uncatalyzed aminolysis reaction in solution? To address this question, the Brønsted coefficient (β_{nuc}) for the α-amino nucleophile was determined on 50S subunits and 70S ribosomes. The Brønsted coefficient reflects the change in charge between the ground state and the transition state of a reaction and thus provides valuable information regarding the reaction trajectory. β_{nuc} was found to be approximately zero, suggesting that the rate of peptidyl transfer is independent of the pK_a of the nucleophilic amine and that the amine remains neutral in the PT transition state. This value is significantly different than observed for uncatalyzed reactions in solution ($\beta_{nuc} = 0.8\text{-}0.9$) where significant positive charge develops on the nucleophile in the transition state. A transition state with an uncharged amine suggests that the degree of N-C bond formation is commensurate with the degree of amine deprotonation. This value excludes mechanisms that require a transition state with significant positive charge development on the α-amine. The data suggest that the ribosome catalyzed reaction is facilitated by amine deprotonation, most likely through a proton shuttle mechanism.

METHODS

Reagents

Amino acids were purchased from CSPS Pharmaceuticals (San Diego, CA, USA). CPmn was purchased from Dharmacon (Lafayette, CO, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA). The cytidine-puromycin β, β -difluorophenylalanyl and the cytidine-puromycin phenylalanyl derivatives syntheses were previously reported(Okuda, Seila et al., 2005).

Puromycin-Phenylalanine and Cytidine-Puromycin-Phenylalanine Derivatives

Detailed descriptions of Pmn(3,4-Cl-Phe), Pmn(3,4-F-Phe), Pmn(4-CH$_3$-Phe) synthesis and characterization is reported in the supplemental section. Amino acids were activated and then coupled to puromycin aminonucleoside (Pmn AN) then deprotected under basic conditions. The synthesis of Pmn(β-F-Phe) will be reported elsewhere. Detailed accounts of CPmn(β, β-F-Phe), CPmn(3,4-Cl-Phe), CPmn(2-F-Phe), CPmn(3,4-F-Phe), CPmn(Phe), CPmn(4-CF$_3$-Phe), CPmn(4-CH$_3$-Phe), CPmn(4-NH$_2$-Phe) synthesis and characterization are reported in the supplemental section. The β-monofluorophenylalanyl (β-F-Phe) amino acid was incompatible with the solid phase chemistry described below due to β-elimination upon addition of base. Following coupling, a succinate linker was introduced at the 2′-oxygen. The derivatives were linked to solid support and a cytidine was added by solid phase chemical synthesis, followed by deprotection under basic conditions.

pK$_a$ Determination

Pmn, Pmn(β, β-F-Phe), and Pmn(Phe) pK_a values were previously reported(Okuda, Seila et al., 2005). The pK_as of the dihydrochloride salt of the puromycin derivatives were determined by monitoring the pH while titrating a 5 mM nucleoside solution in water:methanol:dichloromethane:conc. HCl (4:6:1:1) with 0.1 M NaOH (aq) at 25 °C. Methanol was added to improve the solubility of some derivatives. The measured pK_a values for the synthesized compounds are reported in Figure 1.

Modified 50S Fragment Assay

Large ribosomal subunits were purified from *E. coli* strain MRE600(Seila, Okuda et al., 2005) and the aminolysis rates for the 50S modified fragment assay determined under single

turnover conditions as previously described(Okuda, Seila et al., 2005). The activated 50S ribosomal subunits (final concentration 10 μM) were mixed with labeled CCApcb (<10 nM) and unlabeled aminoacyl substrates at concentrations more than 3-fold above the expected K_M at 25°C. Buffer A (7 mM $MgCl_2$, 7 mM KCl, 150 mM NH_4Cl, 0.1 mM EDTA, 0.2 mM DTT, 25 mM MES, 25 mM MOPS, and 50 mM Tris-HCl pH 8.5 [25 °C]) was used for all 50S assays. Reaction rate (k_{pep}) was determined by plotting the fraction of unreacted CCA-Pcb versus time using Kaleidagraph software and fit to the single exponential curve, fraction unreacted=M1*exp(-(k_{pep}*t))+M2, where M1 is the reaction span, M2 is the endpoint, and t is time. Reactions using each A-site substrate were repeated 2-3 times, and the mean value and standard error were calculated. The log of the average maximum rate values log(k_{pep}) were plotted versus the pK_a for each cytidine puromycin derivative and β_{nuc} value estimated from the slope of the plot by linear regression.

70S Rapid Kinetic Assay

The 70S ribosomes, initiation factors and f[³H]Met-tRNA^fMet were prepared as previously described(Rodnina and Wintermeyer, 1995; Rodnina, Savelsbergh et al., 1999). MFT-mRNA (5′-GGCAAGGAGGUAAAUAAUGUUCACGAUU-3′, underlined sequence codes for fMet-Phe-Thr) was purchased from Dharmacon Research, Inc. (Boulder, CO, USA). Buffer B (50 mM Tris-HCl, 20 mM Bis-Tris, 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$ at pH 8.5 [37 °C]) was used throughout 70S protocols. Initiation complexes were prepared as previously discussed(Katunin, Muth et al., 2002; Beringer, Bruell et al., 2005). Time courses of PT reaction were measured in a quench-flow apparatus (KinTek, Austin, TX, USA). Reaction was started by mixing equal volumes (14 μl) of initiation complexes (0.2 μM) and Pmn(derivative) (1 – 40 mM) at 37 °C. The reaction was quenched by 25% formic acid and f[³H]MetPmn (derivative) was extracted and quantified as previously described(Beringer, Bruell et al., 2005). Pmn(3,4-Cl-Phe), Pmn(3,4-F-Phe), Pmn(4-CH₃-Phe), and Pmn(4-NH₂-Phe) were not soluble in the aqueous buffer, but all compounds were soluble in buffer B that contained 60% DMSO. Kinetic measurements were accomplished in the 60% DMSO buffer B using the entire Pmn derivative series. Saturation could not be achieved for Pmn(β-F-Phe) or Pmn(4-NH₂-Phe). Pmn(β,β-F-Phe), Pmn(β-F-Phe), Pmn, and Pmn(Phe) were sufficiently soluble in buffer B without DMSO, so kinetics were measured with previously published conditions(Katunin, Muth et al., 2002). Single-exponential fitting was used for individual time courses to determine k_{obs} at given concentration of Pmn derivative. Concentration dependencies of k_{obs} were fit to a two-step model where a rapid, reversible binding step is followed by an irreversible reaction to yield k_{pep} values(Katunin, Muth et al., 2002). The Brønsted coefficient was calculated from the two 70S data sets using log(k_{pep}) and pK_a as described above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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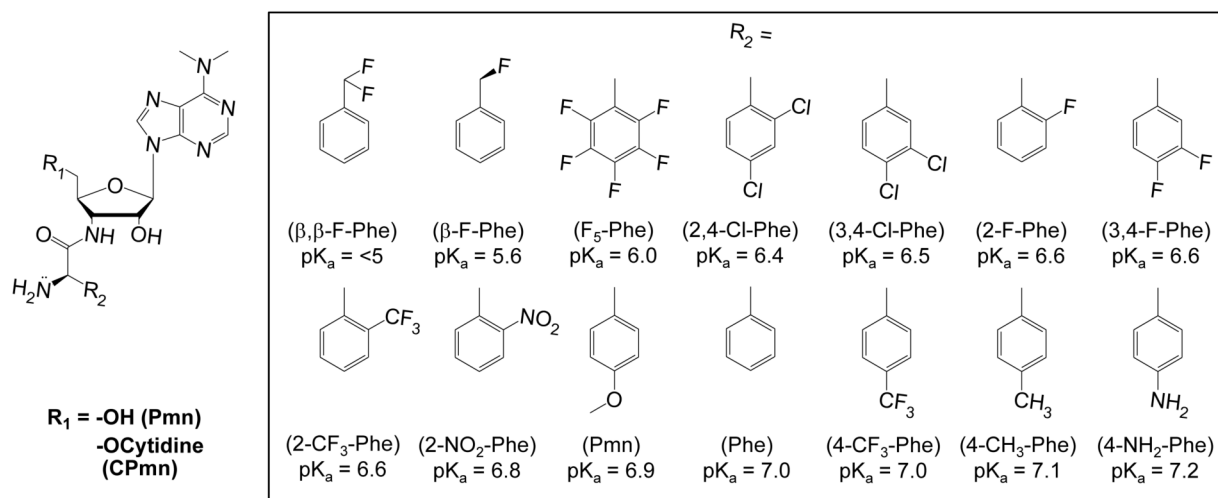
Figure 1.
The series of puromycin derivatives used as A-site substrates.
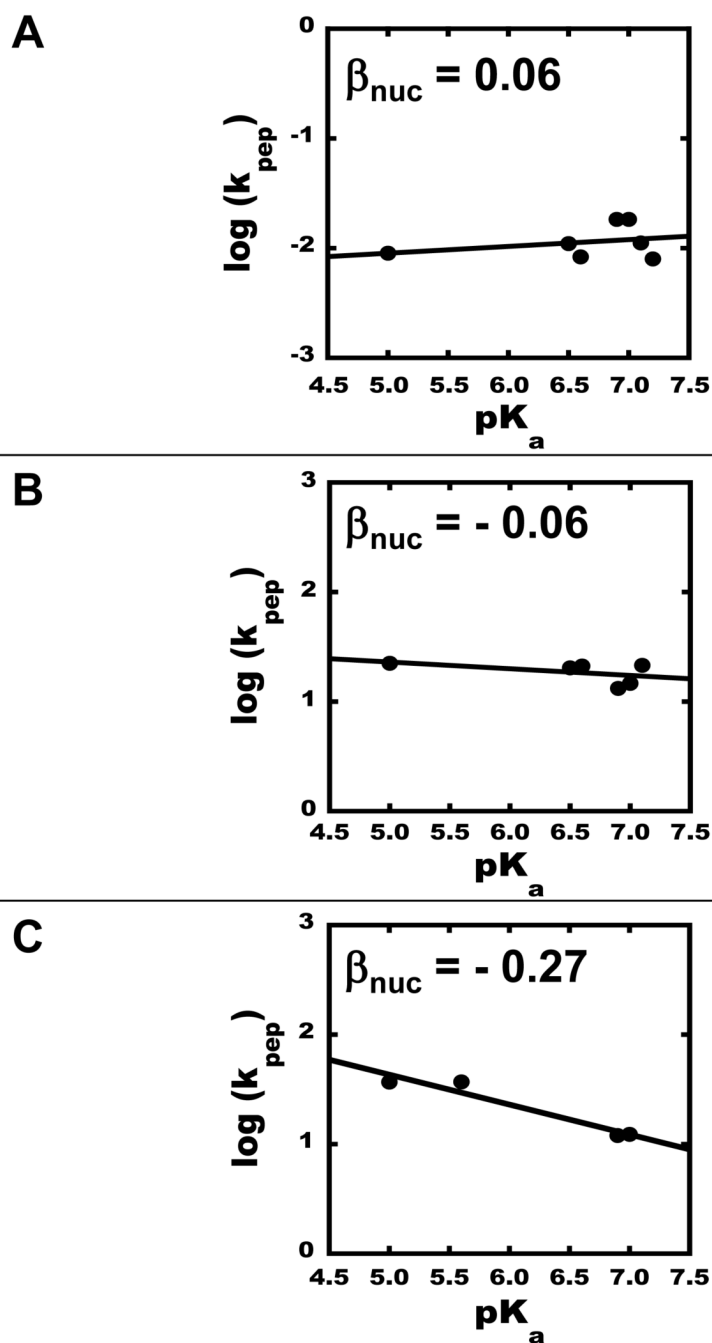
Figure 2.
Brønsted plots for the PT reaction. (A) Brønsted plot of the PT reaction on 50S subunits. The line is the linear regression of the data, $\beta_{nuc} = 0.06$. (B) Brønsted plot of the PT reaction on 70S initiation complexes in the presence of 60% DMSO, with $\beta_{nuc} = -0.06$. (C) Brønsted plot of the PT reaction on 70S initiation complexes in the absence of DMSO, with $\beta_{nuc} = -0.27$.
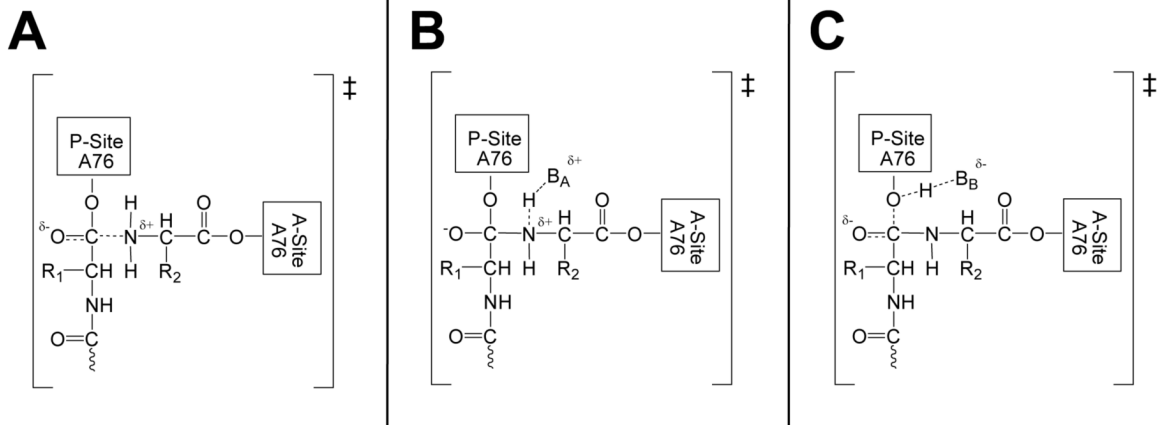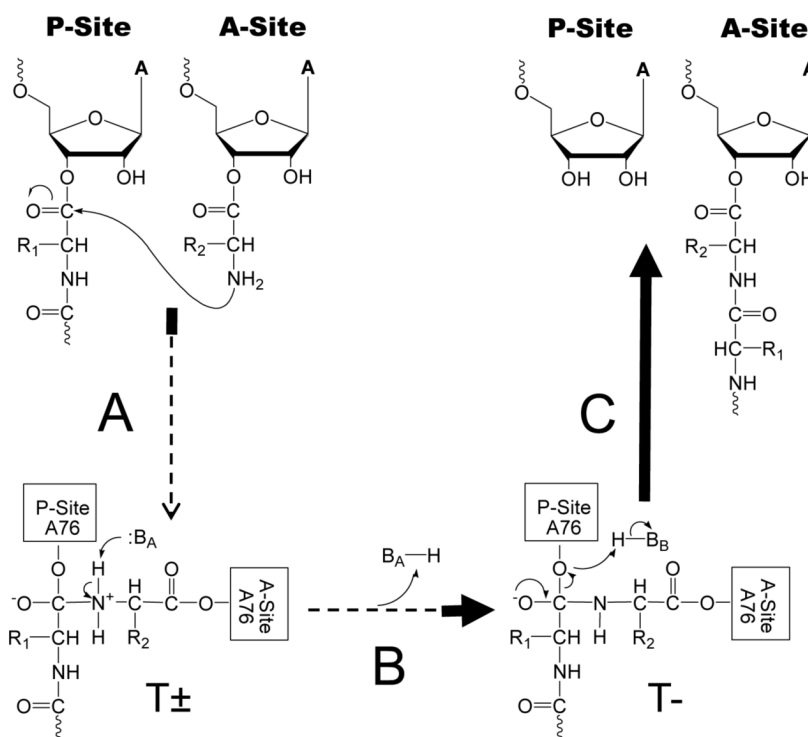
Figure 3.
Stepwise mechanism with two intermediates (T± and T−). The relative energetics of intermediates and TS are arbitrary. The bolded portions of the arrows represent areas where a zero β_{nuc} is consistent for each transition. (A) Possible TS if nucleophilic attack determines the reaction rate. (B) Possible TS if α-amine proton transfer following T± formation determines the reaction rate. (C) Possible TS if T- tetrahedral intermediate breakdown determines the reaction rate.
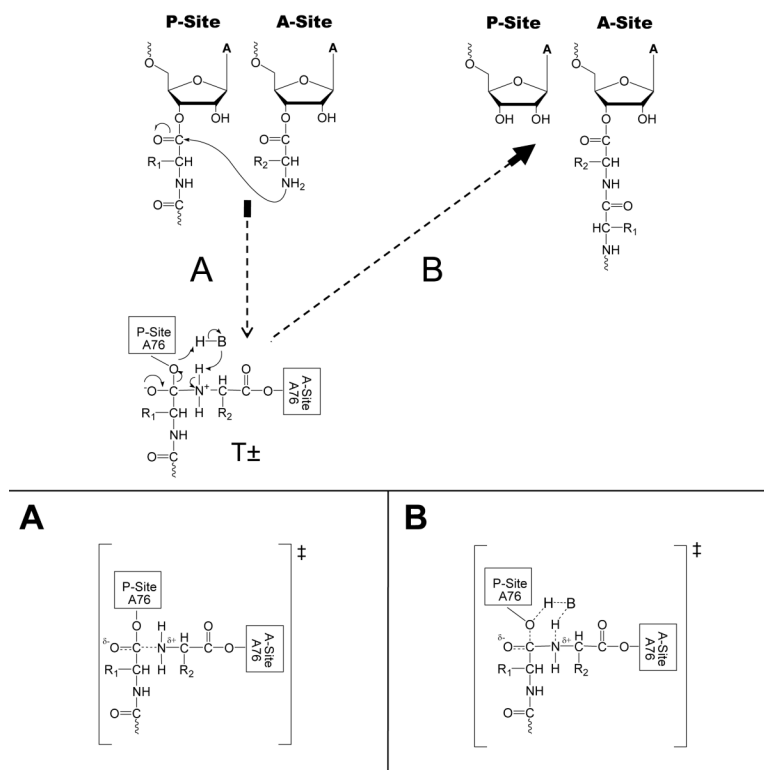
Figure 4.
Stepwise mechanism with one intermediate (T±). The relative energetics of intermediates and TS are arbitrary. The bolded portions of the arrows represent areas where a zero β_{nuc} is consistent for each transition. (A) Possible TS if nucleophilic attack determines the reaction rate. (B) Possible TS if T± tetrahedral intermediate breakdown determines the reaction rate.
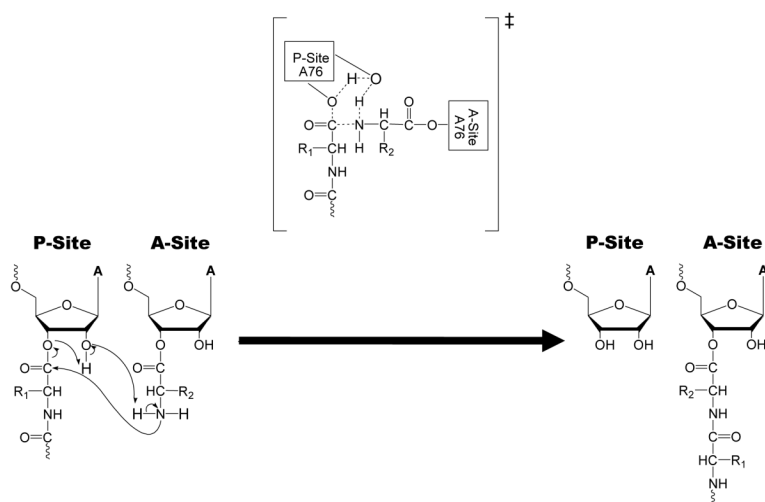
Figure 5.
Fully concerted reaction mechanism. Possible TS if nucleophilic attack, C-O bond cleavage, and proton transfer are concerted in the rate-determining step. The bolded portion of the arrow represents the area where the proposed mechanism is consistent with a zero β_{nuc}.

Table 1

50S modified fragment assay kinetic data (pH 8.5, 25 °C)

	pK_a	k_{pep} (s^{-1})
CPmn(β,β-F-Phe)	<5.0	0.009 ± 0.0003
CPmn(3,4-Cl-Phe)	6.5	0.01 ± 0.01
CPmn(2-F-Phe)	6.6	0.01 ± 0.001
CPmn(3,4-F-Phe)	6.6	0.008 ± 0.0002
CPmn	6.9	0.02 ± 0.001
CPmn(Phe)	7.0	0.02 ± 0.002
CPmn(4-CH$_3$-Phe)	7.1	0.01 ± 0.001
CPmn(4-NH$_2$-Phe)	7.2	0.008 ± 0.001

Table 2

70S assay kinetic data (pH 8.5, 37 °C)

| | pK_a | 60% DMSO | | No DMSO | |
		k_{pep} (s^{-1})	K_M (mM)	k_{pep} (s^{-1})	K_M (mM)
Pmn(β,β-F-Phe)	<5.0	22.4	12.5	36.9	1.3
Pmn(β-F-Phe)	5.6	-	-	37.5	2.9
Pmn(3,4-Cl-Phe)	6.5	20.4	12.7	-	-
Pmn(3,4-F-Phe)	6.6	21.1	12.1	-	-
Pmn	6.9	13.2	6.1	12.0	2.5
Pmn(Phe)	7.0	14.7	8.3	12.3	3
Pmn(4-CH$_3$-Phe)	7.1	21.5	17.9	-	-