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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 with 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 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 (Sievers et al., 2004). Several mechanisms have been proposed to account for this observed rate enhancement, including general acid-base catalysis (Muth et al., 2000; Nissen et al., 2000), catalysis by substrate alignment (Sievers et al., 2004), and substrate-assisted catalysis (Dorner et al., 2003; Weinger et al., 2004; Trobro and Aqvist, 2005). Biochemical, mutational, and recent structural evidence suggest that the general acid-base mechanism, as originally proposed, 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 et al., 2005a, 2005b). Removing the A76 2′-OH decreases the rate of peptide bond formation by at least one million-fold (Weinger 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 et al., 1999; Schmeing et al., 2005a; Rodnina 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 and 0.9 for primary and secondary amines (Bruice and Lapinski, 1958; Jencks and Carriuolo, 1960a; Jencks and Gilchrist, 1968; Satterthwait and Jencks, 1974). Positive values close to 1 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 ($T^{\pm}$ and T^{-}). The α-amine in the first tetrahedral intermediate ($T^{\pm}$) 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 $T^{\pm}$ intermediate (Satterthwait and Jencks, 1974).

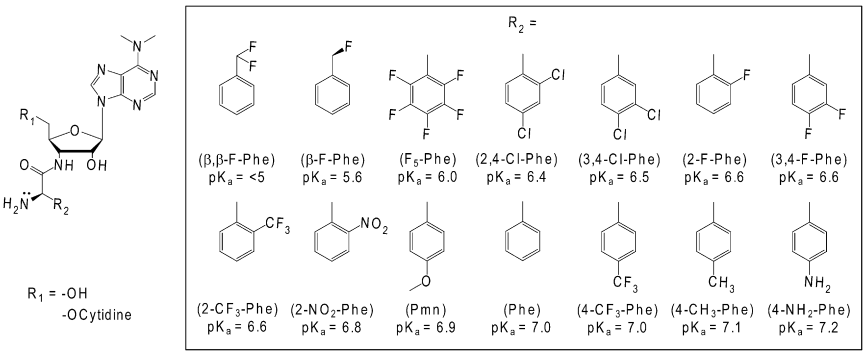

Figure 1. The Series of Pmn Derivatives Used as A-Site Substrates

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-Pmn ($pK_a < 5.0$) reacted 90-fold faster than the phenylalanyl derivative at acidic pH. At higher pHs, the rates of the phenylalanyl and fluorinated phenylalanyl derivatives 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 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 (Pmn) 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 with pK_as ranging from <5 to 7.2 (Figure 1).

β_{nuc} Measurements on the 50S Ribosomal Subunit

The aminolysis rate for each Pmn derivative was determined under single-turnover conditions with the modified 50S fragment assay (Schmeing et al., 2002; Okuda 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 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 (see Figure S1 in the Supplemental Data available with this article online, and 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 binding constant (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, reaction rate (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 by using a single-turnover kinetic assay (Katunin et al., 2002; Beringer 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 et al., 2002). We found that up to 60% DMSO was tolerated, with the only consequence being a slight increase in the K_M for Pmn (Figures S2 and S3).

The rates of PT by the 70S ribosome for all of the Pmn 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)

Table 1. 50S Modified Fragment Assay Kinetic Data

	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
CPmmn(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-H_2-Phe)	7.2	0.008 ± 0.001

Data collected at pH 8.5, 25°C. Values reported are means ± SEM.

and Pmn(4-NH_2-Phe) were 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 3-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 (Figures 2B and 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 analogs (Jencks, 1987).

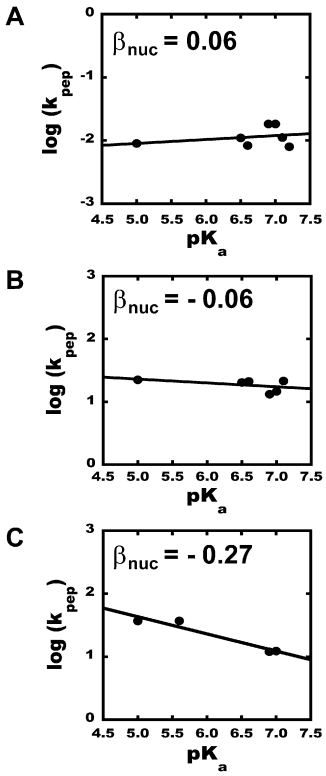

Figure 2. Brønsted Plots for the PT Reaction

(A) Brønsted plot of the PT reaction on 50S subunits. The line is the linear regression of the data, β_{nuc} = 0.06.

(B) Brønsted plot of the PT reaction on 70S initiation complexes in the presence of 60% DMSO, with β_{nuc} = −0.06.

(C) Brønsted plot of the PT reaction on 70S initiation complexes in the absence of DMSO, with β_{nuc} = −0.27.

DISCUSSION

The 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 Pmn-like substrates were synthesized with conservative, nearly isosteric substitutions of the phenylalanine side chain that resulted in pK_as for the α-amino group ranging from <5 to 7.2. Through the use of 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 PT 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 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 nonchemical 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: (1) 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 et al., 2005); (2) kinetic isotope effect (KIE) measured for the A-site substrate indicated that bond formation is at least partially rate limiting (Seila et al., 2005); and (3) a less reactive nucleophile, hydroxypuromycin (the α-amino group is replaced with a hydroxyl) decreased the PT rate at least 20-fold (Seila et al., 2005). For the 70S assay: (1) Pmn binding to the A site is not rate limiting, because peptidyl-tRNA substrates with different peptide lengths react at different rates (e.g., there is a 56-fold difference in the reaction rate of Pmn with fMet-$tRNA^{fMet}$ versus fMetAlaAsnMetPheAla-$tRNA^{Ala}$; Katunin et al., 2002); (2) substitution of Pmn with hydroxypuromycin decreases the rate of PT 200-fold (Katunin et al., 2002); and (3) Pmn binding into the PT center is not rate limiting (Katunin et al., 2002; Sievers et al., 2004). These observations argue that the near-zero β_{nuc} value does not result from monitoring a nonchemical 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, 1960b;

Table 2. 70S Assay Kinetic Data

	60% DMSO			No DMSO	
	pK_a	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	—	—

Data collected at pH 8.5, 37°C.

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 ($T^{\pm}$ and T^{-}) (Jencks and Gilchrist, 1968; Satterthwait and Jencks, 1974; Figure 3). The second is also stepwise, but involves only a single intermediate ($T^{\pm}$) (Figure 4). This is one form of the proton shuttle mechanism, and involves concerted proton transfer with C-O bond cleavage that resolves the $T^{\pm}$ intermediate directly to products (Das et al., 1999; Schmeing et al., 2005a; 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 et al., 2002; Changalov 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 $T^{\pm}$ 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 nonlinear regions of reported Brønsted plots. The nonlinearity of these plots was interpreted to denote a change in the rate-limiting step. Jencks and colleagues 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 $T^{\pm}$ 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 $T^{\pm}$, 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 ($T^{\pm}$) (Figure 4) has two transitions that could limit the overall rate (Das et al., 1999; Schmeing et al., 2005a; Trobro and Aqvist, 2005). This proton shuttle mechanism has been proposed based upon both in silico (Das et al., 1999; Trobro and Aqvist, 2005) and crystallographic studies (Schmeing et al., 2005a). 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 $T^{\pm}$ 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 et al., 2002; Changalov 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 (2007) argued that N-C bond formation is commensurate with α-amine deprotonation based on KIE and Hammett correlation data for the transesterification of 2′/3′-*O*-p-substituted 2′-*O*-trityl adenosines and 2′-deoxyadenosines.

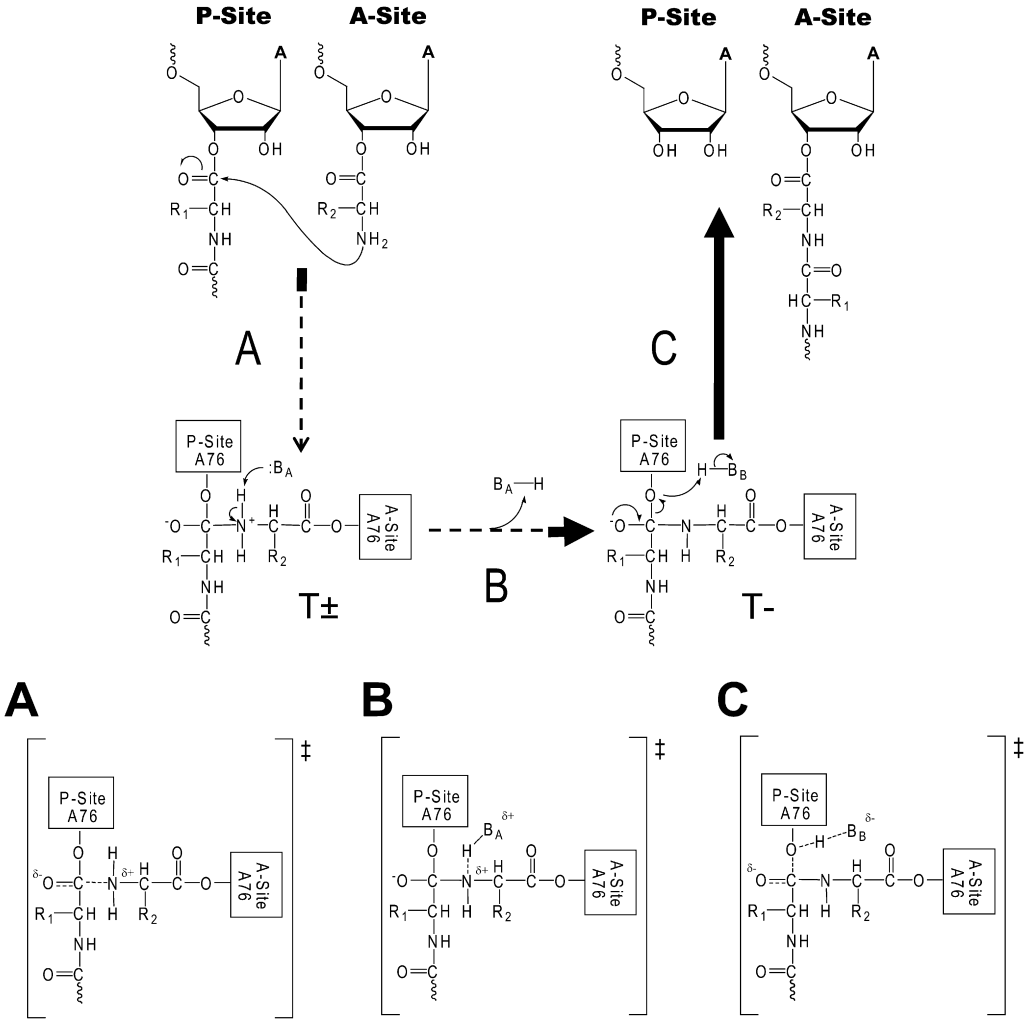

Figure 3. Stepwise Mechanism with Two Intermediates

The bolded portions of the arrows represent areas where a zero β_{nuc} is consistent for each transition. Intermediates are $T^{\pm}$ and T^{-}.

(A) Possible TS if nucleophilic attack determines the reaction rate.

(B) Possible TS if α-amine proton transfer following $T^{\pm}$ formation determines the reaction rate.

(C) Possible TS if T^{-} tetrahedral intermediate breakdown determines the reaction rate.

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 $T^{\pm}$ 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 (K.S. Huang and S.A.S, unpublished data). 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. Komiyama and Bender (1979) 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. Inward and Jencks (1965) 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 that the proton must be removed either before or during the transition state. Finally, a β_{nuc} near zero was measured for amide bond synthesis by acetyltyrosylchymotrypsin. Deuterium

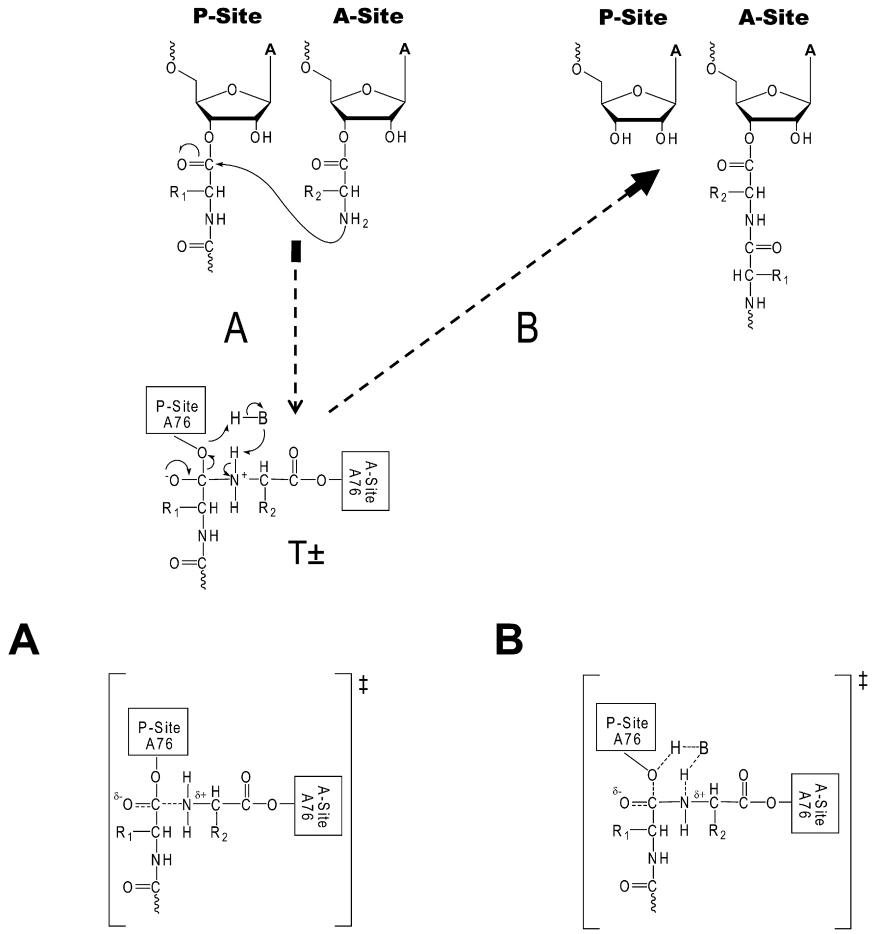

Figure 4. Stepwise Mechanism with One Intermediate

The bolded portions of the arrows represent areas where a zero β_{nuc} is consistent for each transition. Intermediate is $T^{\pm}$.

(A) Possible TS if nucleophilic attack determines the reaction rate.

(B) Possible TS if $T^{\pm}$ tetrahedral intermediate breakdown determines the reaction rate.

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 those 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 (PT) reaction proceed through the same transition state as that observed for a typical uncatalyzed aminolysis reaction in solution? To address this question, the Brønsted coefficient for the α-amino nucleophile (β_{nuc}) 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 PT 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 that

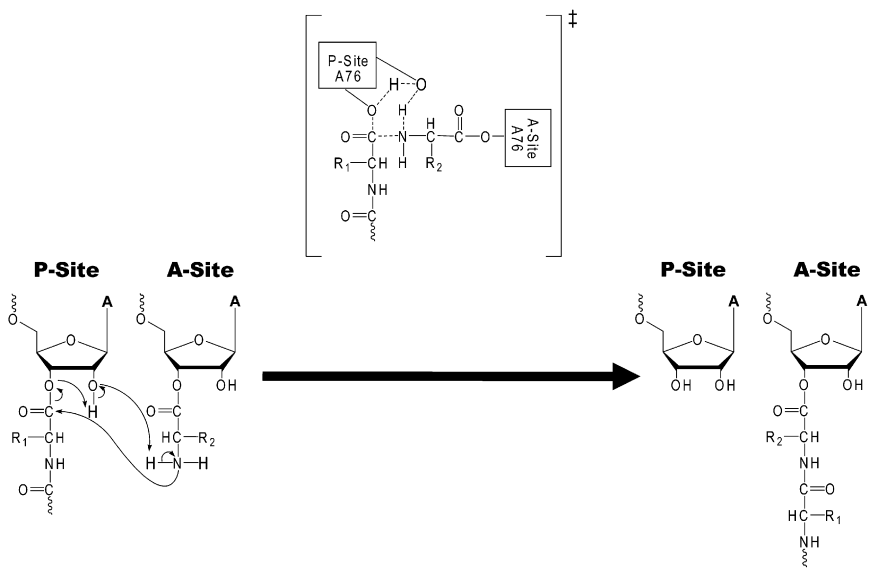

Figure 5. Fully Concerted Reaction Mechanism

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}.

observed for uncatalyzed reactions in solution (β_{nuc} = 0.8–0.9), 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.

EXPERIMENTAL PROCEDURES

Reagents

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

Pmn-Phenylalanine and Cytidine-Pmn-Phenylalanine Derivatives

Detailed descriptions of Pmn(3,4-Cl-Phe), Pmn(3,4-F-Phe), and Pmn(4-CH_3-Phe) synthesis and characterization are reported in the Supplemental Data. Amino acids were activated and then coupled to Pmn aminonucleoside, 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), and CPmn(4-NH_2-Phe) synthesis and characterization are reported in the Supplemental Data. 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 have previously been reported (Okuda et al., 2005). The pK_as of the dihydrochloride salt of the Pmn derivatives were determined by monitoring the pH while titrating a 5 mM nucleoside solution in water:methanol:dichloromethane:concentrated 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 *Escherichia coli* strain MRE600 (Seila et al., 2005), and the aminolysis rates for the 50S modified fragment assay were determined under single-turnover conditions, as previously described (Okuda 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 greater than 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. k_{pep} was determined by plotting the fraction of unreacted CCA-Pcb versus time with 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 two to three 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 Pmn derivative and the β_{nuc} value was estimated from the slope of the plot by linear regression.

70S Rapid Kinetic Assay

The 70S ribosomes, initiation factors and f[^{3}H]Met-$tRNA^{fMet}$ were prepared as previously described (Rodnina and Wintermeyer, 1995; Rodnina et al., 1999). MFT-mRNA (5′-GGCAAGGAGGUAAAUAAUGUUCACGAUU-3′, underlined sequence codes for fMet-Phe-Thr) was purchased from Dharmacon Research, Inc. (Boulder, CO). Buffer B (50 mM Tris-HCl, 20 mM Bis-Tris, 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$ [pH 8.5], 37°C) was used throughout 70S protocols. Initiation complexes were prepared as previously discussed (Katunin et al., 2002; Beringer et al., 2005). Time courses of PT reaction were measured in a quench-flow apparatus (KinTek, Austin, TX). 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[^{3}H]MetPmn(derivative) was extracted and quantified as previously described(Katunin et al., 2002). Pmn(3,4-Cl-Phe), Pmn(3,4-F-Phe), Pmn(4-CH_3-Phe), and Pmn(4-NH_2-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 with the entire Pmn derivative series. Saturation could not be achieved for Pmn(β-F-Phe) or Pmn(4-NH_2-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 et al., 2002). Single-exponential fitting was used for individual time courses to determine k_{obs} at given concentrations of Pmn derivative. Concentration dependencies of k_{obs} were fit to a two-step model in which a rapid, reversible binding step is followed by an irreversible reaction to yield k_{pep} values (Katunin et al., 2002). The Brønsted coefficient was calculated from the two 70S data sets with log(k_{pep}) and pK_a, as described above.

SUPPLEMENTAL DATA

Supplemental Data, including three figures and Supplemental Experimental Procedures used in this work, are available online at http://www.chembiol.com/cgi/content/full/15/5/493/DC1/.

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