

# Transfer RNA Modulates the Editing Mechanism Used by Class II Prolyl-tRNA Synthetase\*

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Aminoacyl-tRNA synthetases catalyze the attachment of amino acids to their cognate tRNAs. To prevent errors in protein synthesis, many synthetases have evolved editing pathways by which misactivated amino acids (pre-transfer editing) and misacylated tRNAs (post-transfer editing) are hydrolyzed. Previous studies have shown that class II prolyl-tRNA synthetase (ProRS) possesses both pre- and post-transfer editing functions against noncognate alanine. To assess the relative contributions of pre- and post-transfer editing, presented herein are kinetic studies of an *Escherichia coli* ProRS mutant in which post-transfer editing is selectively inactivated, effectively isolating the pre-transfer editing pathway. When post-transfer editing is abolished, substantial levels of alanine mischarging are observed under saturating amino acid conditions, indicating that pre-transfer editing alone cannot prevent the formation of Ala-tRNA<sup>Pro</sup>. Steady-state kinetic parameters for aminoacylation measured under these conditions reveal that the preference for proline over alanine is 2000-fold, which is well within the regime where editing is required. Simultaneous measurement of AMP and Ala-tRNA<sup>Pro</sup> formation in the presence of tRNA<sup>Pro</sup> suggested that misactivated alanine is efficiently transferred to tRNA to form the mischarged product. In the absence of tRNA, enzyme-catalyzed Ala-AMP hydrolysis is the dominant form of editing, with “selective release” of noncognate adenylate from the active site constituting a minor pathway. Studies with human and *Methanococcus jannaschii* ProRS, which lack a post-transfer editing domain, suggest that enzymatic pre-transfer editing occurs within the aminoacylation active site. Taken together, the results reported herein illustrate how both pre- and post-transfer editing pathways work in concert to ensure accurate aminoacylation by ProRS.

Aminoacyl-tRNA synthetases (AARS)<sup>3</sup> catalyze the acylation of tRNAs with cognate amino acids via a two-step mecha-

nism (1–3). In the first step, an amino acid is activated with ATP to form an aminoacyl-adenylate (AA-AMP) intermediate. In the second step, the amino acid is transferred to the 3' end of the cognate tRNA to form the aminoacylated tRNA product. Therefore, the fidelity of protein synthesis is maintained, in large part, by the selectivity of the AARS for their specific substrates. The selection of cognate tRNA is based upon a number of unique identity elements within the nucleic acid sequence, resulting in high specificity of AARS for their cognate tRNAs (4). However, selection of cognate amino acids poses a greater challenge because the amino acid is a smaller molecule with fewer distinguishing features (5). Misactivation of amino acids that are smaller than or isosteric with the cognate amino acid is often observed. These errors may result in mischarged tRNA products and subsequent incorporation of incorrect amino acids into proteins, thereby challenging the viability or function of living cells (6).

To correct for errors in amino acid selection, many AARS have evolved a series of editing mechanisms (Scheme 1) (7). In pre-transfer editing, the noncognate AA-AMP is hydrolyzed to yield free amino acid and AMP (paths 1–3). This reaction can occur via tRNA-dependent or -independent enzymatic hydrolysis in either the synthetic active site or at a separate editing site (paths 1 and 3) or via “selective release” of the enzyme-bound AA-AMP into solution and subsequent hydrolysis (path 2). Post-transfer editing consists of the hydrolysis of mischarged aminoacyl-tRNA at a separate domain on the enzyme (path 4). Of the 20 AARS, ten enzymes representative of both classes have been observed to exhibit editing activities (8, 9).

Prolyl-tRNA synthetase (ProRS) is a class II synthetase that has been shown to misactivate a variety of both natural and unnatural, noncognate amino acids and to possess multiple mechanisms to maintain fidelity (10–16). Post-transfer editing of Ala-tRNA<sup>Pro</sup> by bacterial ProRS is carried out by a large insertion domain located between class II consensus motifs 2 and 3 (12). Furthermore, the insertion domain, when expressed as an independent protein, maintains its deacylation activity, and this activity can be modulated via mutation of the highly conserved Lys<sup>279</sup> residue (13). Recent work has identified a variety of homologs of the insertion domain that also possess deacylation function. In particular, *Haemophilus influenzae* YbaK was recently shown to be specific for the deacylation of Cys-tRNA<sup>Pro</sup>, providing a mechanism by which mischarged cysteine is cleared in some organisms (16–19).

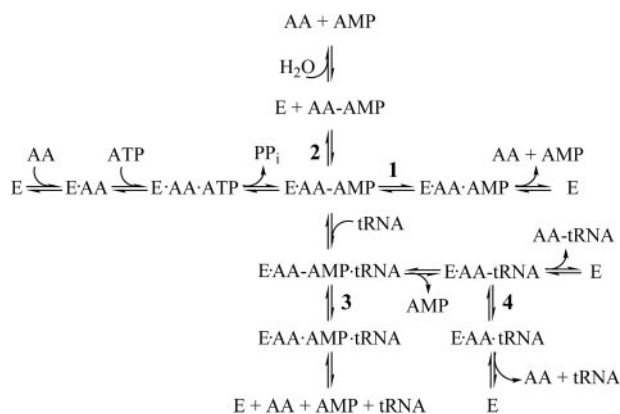
Although overwhelming evidence exists for post-transfer editing by bacterial ProRS, it may not be the dominant mechanism. In the absence of tRNA, alanine stimulates ATP hydroly-

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<sup>3</sup> The abbreviations used are: AARS, aminoacyl-tRNA synthetase(s); AA-AMP, aminoacyl-adenylate; Ala-AMP, alanyl-adenylate; ProRS, prolyl-tRNA synthetase; Ec, *E. coli*; Mj, *M. jannaschii*; Hm, human; WT, wild type; PP<sub>1</sub>ase, inorganic pyrophosphatase.



SCHEME 1. Minimal kinetic scheme of enzyme catalysis by aminoacyl-tRNA synthetases including pre-transfer (paths 1–3) and post-transfer (path 4) editing pathways.

ysis (10), indicative of either enzymatic pre-transfer editing (5) and/or release of the noncognate adenylate into solution (14). Whereas the assessment of pre-transfer editing in the absence of tRNA is straightforward, the presence of tRNA complicates the analysis of pre-transfer editing because of the concurrent hydrolysis of mischarged Ala-tRNA<sup>Pro</sup>, allowing only for the extent of *total* editing to be measured. To directly assess pre-transfer editing in the course of the aminoacylation reaction, we have kinetically characterized the K279A *Escherichia coli* (Ec) ProRS mutant that is defective in post-transfer editing (12), thereby effectively isolating the pre-transfer pathway. Characterization of this simplified reaction scheme allows for the direct assessment of the relative contributions of pre- and post-transfer editing to the fidelity of aminoacylation by Ec ProRS, revealing that both pathways work in concert to ensure efficient aminoacylation. Studies carried out with human (Hm) and *Methanococcus jannaschii* (Mj) ProRS, which lack post-transfer editing activity, were consistent with results obtained using the bacterial system and show that enzymatic pre-transfer editing is maintained even in the absence of the post-transfer editing domain.

## EXPERIMENTAL PROCEDURES

**Enzyme Preparation**—Wild type (WT) and K279A Ec ProRS (12, 20), Hm ProRS (21), and Mj ProRS (22) were prepared as described previously. Enzyme concentrations were determined by active site titration (23).

**RNA Preparation**—Ec tRNA<sup>Pro</sup> was prepared by *in vitro* transcription using BstNI-linearized plasmid as described previously (24). tRNA<sup>Pro</sup> was labeled with <sup>32</sup>P at the 3'-terminal internucleotide linkage via published procedures with slight modification (25). A 100- $\mu$ l reaction containing 50 mM glycine-HCl buffer, pH 9.0, 10 mM MgCl<sub>2</sub>, 1  $\mu$ M tRNA<sup>Pro</sup>, 0.82  $\mu$ M [ $\alpha$ -<sup>32</sup>P]ATP (Amersham Biosciences), 50  $\mu$ M sodium PP<sub>i</sub>, and 30  $\mu$ g/ml tRNA-terminal nucleotidyl transferase was incubated for 5 min at 37 °C. Yeast pyrophosphatase (PP<sub>i</sub>ase) was added to a final concentration of 10 units/ml, and the reaction was incubated for an additional 30 s. The reaction was quenched by phenol/chloroform extraction, filtered through a G25 microspin column (Amersham Biosciences) to remove unreacted nucleotides, and isolated via ethanol precipitation. Purity was

checked by denaturing polyacrylamide gel electrophoresis, revealing successful removal of free nucleotides.

Nonchargeable tRNA was obtained by periodate oxidation. Briefly, a 50- $\mu$ l reaction containing 50 mM NaOAc, pH 5, 2.5 mM sodium periodate, and 80  $\mu$ M tRNA<sup>Pro</sup> was incubated at 37 °C for 60 min. Glucose was added to a final concentration of 50 mM, and the reaction was incubated at 0 °C for an additional 1.5 h. The oxidized product was collected via ethanol precipitation. Charging assays utilizing [<sup>3</sup>H]Pro conducted using published conditions (26, 27) detected no aminoacylated product.

**Enzyme Assays**—Aminoacylation assays to determine kinetic parameters and charging levels were performed at 37 °C. The reactions (20  $\mu$ l) contained 50 mM HEPES, pH 7.5, 20 mM KCl, 25 mM MgCl<sub>2</sub>, 4 mM ATP, 0.5 mM dithiothreitol, 20  $\mu$ M tRNA<sup>Pro</sup>, trace amounts of <sup>32</sup>P-labeled tRNA<sup>Pro</sup>, and variable levels of amino acid. The reactions were initiated upon addition of Ec K279A ProRS to a final concentration of 0.1  $\mu$ M (proline) or 0.5  $\mu$ M (alanine), unless otherwise specified. At the desired time point, a 1.5- $\mu$ l aliquot was removed and quenched into 4  $\mu$ l of 0.2 M NaOAc, pH 5, containing 0.4 unit/ $\mu$ l of nuclease P1 (Sigma) at 0 °C. Upon completion of the assay, the aliquots were digested at room temperature for 15 min. Aliquots (1  $\mu$ l) were spotted onto polyethyleneimine cellulose plates (Sigma) pre-washed with water. Separation of [<sup>32</sup>P]AMP and aminoacyl-<sup>32</sup>P]AMP was performed by developing TLC plates in 0.1 M ammonium acetate, 5% acetic acid. The plates were visualized by phosphorimaging, and the data were analyzed using Bio-Rad Molecular Imager FX software. The fraction of acylated tRNA was determined from the ratio of aminoacyl-AMP to the total amount of AMP in the reaction aliquot. Kinetic parameters were determined using at least five concentrations for each amino acid, ranging from 0.01 to 1 mM for proline and from 10 to 450 mM for alanine. The data were analyzed using Lineweaver-Burk plots.

TLC assays to measure the rate of AMP formation were performed as described previously (14), with the exception that pyrophosphatase was omitted from the reaction. All of the assays contained either 3 mM proline or 450 mM alanine, 250  $\mu$ M ATP, 100 mM Tris-HCl, pH 7.0, 10 mM KF, 10 mM 2-mercaptoethanol, 10 mM MgCl<sub>2</sub>, and 0.25  $\mu$ M [ $\alpha$ -<sup>32</sup>P]ATP. The reactions were performed at 37 °C and initiated with either 0.5  $\mu$ M Ec K279A ProRS or 5  $\mu$ M WT Ec ProRS, Hm ProRS, or Mj ProRS. The data were analyzed as described previously at 0.5  $\mu$ M enzyme concentrations (14). At 5  $\mu$ M enzyme, the rates were obtained using only the initial time points, where the plot of [AMP] versus time was linear. The data were fit to the following equation:

$$y = B + k_{ss}t \quad (\text{Eq. 1})$$

where  $B$  is the burst amplitude and  $k_{ss}$  is the steady-state rate. The reaction rate constants ( $k_{obs}$ ) were calculated by dividing the steady-state rate of the reaction (in  $\mu$ M/s) by the total enzyme concentration ( $\mu$ M). To directly compare the rates of mischarging with AMP formation, charging assays were performed utilizing the same reaction conditions as described for the assay of AMP formation. Digestion of tRNA and subsequent data analysis were as described above.

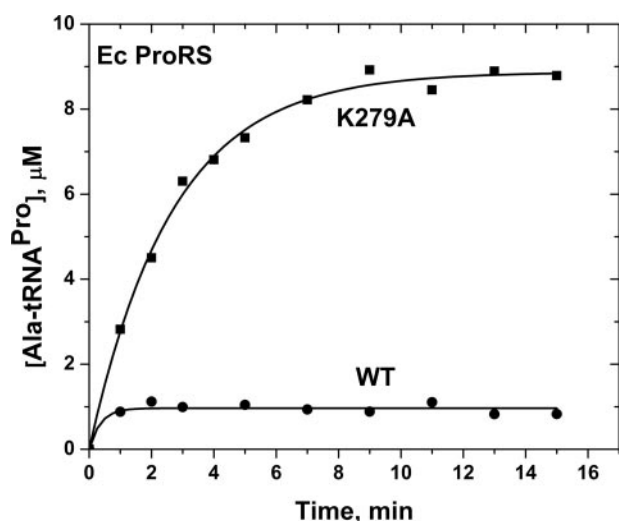


FIGURE 1. Formation of mischarged Ala-tRNA<sup>Pro</sup> by 1  $\mu\text{M}$  WT (●) and K279A (■) Ec ProRS in the presence of 450 mM alanine and 20  $\mu\text{M}$  tRNA<sup>Pro</sup>. The conditions are as described under "Experimental Procedures."

## RESULTS

**Characterization of Alanine Mischarging by a Mutant ProRS That Lacks Post-transfer Editing Activity**—In this work, we kinetically characterized the EC K279A ProRS mutant, in which post-transfer editing was previously shown to be essentially abolished (12). By focusing on this enzyme, the impact that pre-transfer editing has upon alanine charging kinetics could be assessed. Initial steady-state charging assays performed with proline revealed that both WT and K279A ProRS charge the cognate amino acid substrate with equal efficiency (data not shown), indicating that the fidelity of the synthetic active site in this mutant is maintained.

To characterize the alanine charging kinetics for both WT and K279A ProRS, we used an assay in which the 3'-terminal internucleotide linkage of tRNA<sup>Pro</sup> is labeled with [<sup>32</sup>P] (25). Importantly, use of this assay allows for kinetic characterization at saturating amino acid levels, in contrast to the more commonly used trichloroacetic acid precipitation method, which uses <sup>3</sup>H-labeled amino acids (12, 26). At saturating alanine levels (450 mM), the post-transfer editing-deficient K279A ProRS shows that substantial alanine mischarging capabilities and plateau level charging can be achieved (Fig. 1). In contrast, WT ProRS shows detectable but low mischarging levels, because of post-transfer editing of mischarged alanine, as previously reported (12). A plateau level is observed because of a dynamic equilibrium between mischarging and hydrolysis. Thus, abolishing post-transfer editing results in an increase in mischarged tRNA, implying that pre-transfer editing alone cannot ensure the fidelity of aminoacyl-tRNA<sup>Pro</sup> synthesis.

To further quantify this effect, we determined steady-state parameters for the two-step aminoacylation reaction for both cognate proline and noncognate alanine by Ec K279A ProRS. As shown in Table 1, the values measured when post-transfer editing is abolished result in a discrimination factor for proline over alanine of 1 in 1900, which is well within the range where editing is needed (28), even when the relative amino acid concentrations are not taken into account. In *E. coli*, alanine is present at a higher concentration than proline (148  $\mu\text{M}$  versus 9

TABLE 1

Kinetic parameters for the aminoacylation of tRNA<sup>Pro</sup> with proline and alanine by K279A Ec ProRS

The parameters were determined at pH 7.5 via Lineweaver-Burk analysis in the presence of varying amino acid concentrations, as described under "Experimental Procedures."

Amino acid	$k_{\text{cat}}$ $s^{-1}$	$K_m$ $\text{mM}$	$k_{\text{cat}}/K_m$ $s^{-1} \text{mM}^{-1}$	$k_{\text{cat}}/K_m$ (relative)
Pro	$0.23 \pm 0.08$	$0.15 \pm 0.02$	1.5	1
Ala	$0.024 \pm 0.002$	$31 \pm 2$	$7.7 \times 10^{-4}$	$5.1 \times 10^{-4}$

TABLE 2

Kinetic parameters of AMP and AA-AMP formation and aminoacylation by K279A Ec ProRS

The rate constants were measured at pH 7.0 using 0.5  $\mu\text{M}$  ProRS as described under "Experimental Procedures" unless noted otherwise.

	$k_{\text{obs}}$ (K279A)	
	Pro	Ala
	$s^{-1}$	
AMP, no tRNA	— <sup>a</sup>	$(3.4 \pm 0.7) \times 10^{-2}$
AMP, +tRNA	$0.16 \pm 0.03$	$(5.0 \pm 0.2) \times 10^{-2}$
Ala-AMP, no tRNA	ND <sup>b</sup>	$(7 \pm 3) \times 10^{-3}$
Ala-AMP, +tRNA	ND	$(6.8 \pm 0.8) \times 10^{-3}$
Aminoacylation	$0.35 \pm 0.08^c$	$(5.7 \pm 1.3) \times 10^{-2}$

<sup>a</sup> The dash denotes a reaction in which no product was detected.

<sup>b</sup> ND denotes "not determined."

<sup>c</sup> Aminoacylation in the presence of cognate proline was performed using 0.1  $\mu\text{M}$  ProRS as described under "Experimental Procedures."

$\mu\text{M}$ , respectively) (29), which effectively reduces the discrimination factor to 1 in 120. These results further support the conclusion that pre-transfer editing is not sufficient to prevent the mischarging of alanine in the presence of tRNA.

**Measurement of AMP Formation in the Presence of tRNA**—As shown in Scheme 1, both pre-transfer editing and amino acid transfer to form charged tRNA result in the release of AMP. In the absence of editing, the formation of AMP is expected to be stoichiometric with respect to the formation of charged tRNA. In the presence of editing, AMP is formed to a greater extent than charged tRNA, because both adenylate hydrolysis (pre-transfer editing) and mischarging and subsequent deacylation (post-transfer editing) result in the production of AMP. Because post-transfer editing is abolished in the case of K279A ProRS, nonstoichiometric production of AMP reports directly on the extent of pre-transfer editing.

To assess the formation of AMP as both a function of pre-transfer editing and of tRNA charging, we monitored steady-state AMP formation and tRNA charging with K279A Ec ProRS under the same conditions, in the presence of either proline or alanine. Importantly, all of the assays were performed in the absence of PP<sub>i</sub>ase, which prevents pyrophosphorolysis of the alanyl-adenylate. Thus, under these conditions, the reverse reaction may also take place. For cognate proline, no appreciable formation of AMP by K279A ProRS is detected in the absence of tRNA, consistent with the lack of cognate amino acid editing (Table 2). When the overall accuracy of the experimental technique and standard deviations are taken into account, the presence of tRNA results in a rate of AMP formation that agrees well with the rate of tRNA aminoacylation under identical conditions (Table 2). Similar results have been demonstrated upon formation of His-tRNA<sup>His</sup> by Ec histidyl-tRNA synthetase (30).

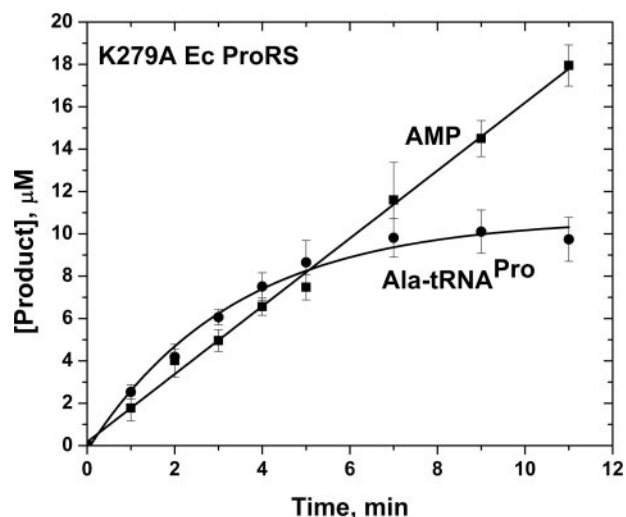


FIGURE 2. Reaction time courses showing the formation of AMP in the presence of tRNA<sup>Pro</sup> (■) and of Ala-tRNA<sup>Pro</sup> (●) by K279A Ec ProRS (0.5 μM). The experimental conditions were identical for both assays, as described under "Experimental Procedures."

TABLE 3

Steady-state kinetic parameters of AMP and AA-AMP formation by Ec, Hm, and Mj ProRS

All of the kinetic parameters were determined under burst conditions at pH 7.0 using 5 μM ProRS as described under "Experimental Procedures."

	BA <sup>a</sup>		k <sub>obs</sub>	
	AMP	AA-AMP	AMP	AA-AMP
	μM		s <sup>-1</sup>	
Ec ProRS (Ala)	12 ± 7	0.7 ± 0.6	(3.0 ± 0.9) × 10 <sup>-2</sup>	(5 ± 1) × 10 <sup>-3</sup>
Ec ProRS (Pro)	6 ± 2	0.2 ± 0.02	(2.2 ± 0.7) × 10 <sup>-3</sup>	(8.2 ± 0.4) × 10 <sup>-5</sup>
Mj ProRS (Ala)	10 ± 3	1 ± 0.3	(1.5 ± 0.4) × 10 <sup>-2</sup>	(2.4 ± 0.7) × 10 <sup>-3</sup>
Hm ProRS (Ala)	2 ± 5	— <sup>b</sup>	(6.1 ± 0.4) × 10 <sup>-2</sup>	—

<sup>a</sup> BA is the burst amplitude extrapolated to time 0.

<sup>b</sup> The dash denotes a reaction in which no product was detected.

As shown in Fig. 2 and Tables 2 and 3, the steady-state rate of noncognate alanine mischarging is equal to the steady-state rate of AMP formation in the presence and absence of tRNA. Furthermore, when the supply of chargeable tRNA is exhausted (*i.e.* plateau is reached), AMP formation continues with the same steady-state rate, presumably because of adenylate hydrolysis. The observation of similar rates for both reactions in the presence and absence of tRNA implies that they share a common rate-limiting step. Furthermore, these data suggest that the transfer step is kinetically fast enough to out-compete pre-transfer editing (see also Fig. 1).

In some AARS, it has been demonstrated that the presence of tRNA can induce a conformational change that impacts the synthetic active site. For example, GlnRS is catalytically inactive prior to binding tRNA<sup>Gln</sup>, demonstrating the importance of tRNA-induced conformational changes (31). Similarly, mutant tRNA<sup>His</sup> has been shown to alter the kinetics of His-AMP formation by HisRS (30). To probe the possibility that the binding of tRNA induces a conformational change that stabilizes the enzyme-bound Ala-AMP intermediate and thereby increases the observed rate of mischarging, we measured the rate of AMP formation by K279A Ec ProRS in both the presence and absence of a nonchargeable tRNA substrate. If bound tRNA is capable of stabilizing the adenylate intermediate and decreasing the rate

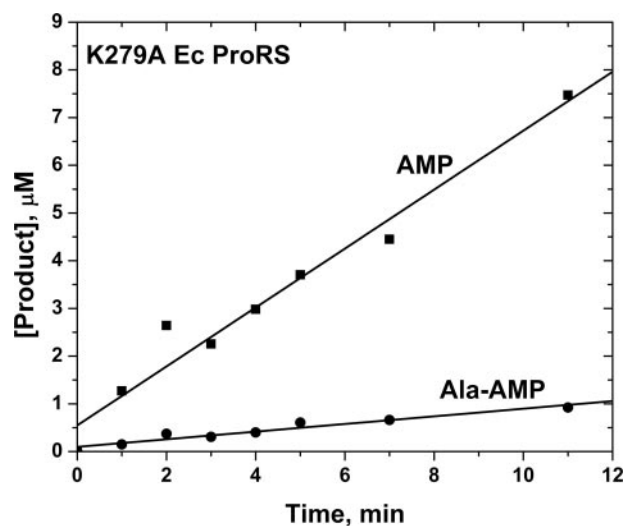


FIGURE 3. Reaction time courses showing the formation of AMP (■) and Ala-AMP (●) in the absence of tRNA<sup>Pro</sup> using 0.5 μM K279A Ec ProRS. The experiments were performed as described under "Experimental Procedures."

of editing, a reduced rate of AMP formation should be observed, because in this case AMP formation can only occur via pre-transfer editing. However, in the presence of periodate-treated tRNA, the rate of AMP formation was measured to be 0.048 s<sup>-1</sup>, which is similar to the observed rate in the absence of tRNA (Table 2). We therefore conclude that the binding of tRNA does not increase the stability of Ala-AMP, implying that alanine transfer to tRNA<sup>Pro</sup> can kinetically compete with adenylate hydrolysis. Furthermore, the addition of tRNA does not further stimulate AMP production beyond the level formed during aminoacylation, consistent with the earlier observation that tRNA does not stimulate pre-transfer editing by ProRS (10).

**Enzyme-catalyzed Hydrolysis of Ala-AMP**—Although the results described above clearly point to post-transfer editing as the dominant mechanism in the presence of chargeable tRNA, in the absence of tRNA, Ala-AMP is hydrolyzed at a significant rate (0.034 s<sup>-1</sup>). Consistent with our previous studies of WT Ec ProRS (14), the steady-state rate of AMP formation by K279A ProRS in the presence of alanine is significantly faster (5- and 7-fold in the absence and presence of tRNA, respectively) than the steady-state rate of Ala-AMP formation (Fig. 3 and Table 2). We also previously reported that the rate of AMP formation by WT ProRS in the presence of alanine was ~2-fold slower than the rate of nonenzymatic hydrolysis of Ala-AMP (~0.1 min<sup>-1</sup>) and therefore concluded that the majority of pre-transfer editing was occurring via the selective release pathway rather than via enzymatic hydrolysis (14). However, our present studies of K279A ProRS show that the rate of AMP formation in the presence of alanine is 0.034 s<sup>-1</sup>, which is at least 20 times faster than the rate of nonenzymatic hydrolysis (32) and suggests that pre-transfer editing is enzyme catalyzed. These results motivated us to revisit our previous conclusions with WT ProRS, and in doing so, a calculation error was uncovered.<sup>4</sup> We now report a

<sup>4</sup> In calculating the rate of AMP formation reported in the first row of Table 3 in Ref. 14, we inadvertently divided the amount of AMP formed per min by the concentration of ATP used instead of by the enzyme concentration. The revised numbers are: k<sub>obs</sub> = 9.6 min<sup>-1</sup> for proline and 27.2 min<sup>-1</sup> for alanine.

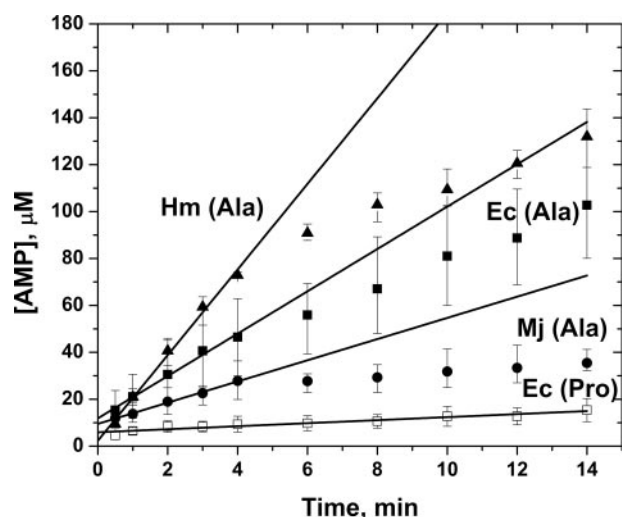


FIGURE 4. Time course of AMP formation under burst conditions using  $5 \mu\text{M}$  WT Ec ProRS in the presence of alanine (■) and proline (□),  $5 \mu\text{M}$  Hm ProRS in the presence of alanine (▲), and  $5 \mu\text{M}$  Mj ProRS in the presence of alanine (●). All of the reactions were performed in the absence of  $\text{tRNA}^{\text{Pro}}$  as described under "Experimental Procedures."

revised rate of  $0.45 \text{ s}^{-1}$  for overall AMP formation in the presence of alanine by WT ProRS. This rate is 240 times faster than the rate of nonenzymatic hydrolysis. Although this recalculated rate is significantly larger than what we report herein for WT ProRS (Table 3), we attribute this difference to the presence of  $\text{PP}_i$ ase in our previous studies. Inclusion of  $\text{PP}_i$ ase in the reaction mixture will eliminate the reverse reaction and result in a higher net rate of AMP formation.

Therefore, the fast formation of AMP in the absence of tRNA is attributed to enzyme-catalyzed hydrolysis of the alanyl-adenylate both in the case of WT and K279A ProRS. However, because the concentration of Ala-AMP is observed to be higher than the enzyme concentration (Fig. 3), we conclude that a small portion of adenylate is released from the active site, and the relatively slow rate of nonenzymatic hydrolysis allows for a build-up of Ala-AMP over time. In the absence of tRNA, based upon the ratio of AMP formation to adenylate formation reported in Table 2, we estimate that 80% of the adenylate is hydrolyzed enzymatically, establishing this mechanism as the dominant form of pre-transfer editing. Similarly, in the presence of tRNA, where AMP production coincides with the formation of Ala- $\text{tRNA}^{\text{Pro}}$ , <15% of the adenylate is released, with the majority converted to the mischarged Ala- $\text{tRNA}^{\text{Pro}}$  product (Table 2). Thus, both in the absence and presence of tRNA, selective release constitutes a minor pathway for editing ( $\leq 20\%$ ).

To quantify the amount of AMP and AA-AMP formed during the first enzyme turnover, steady-state rates of AMP formation were also measured using higher WT Ec ProRS concentrations ( $5 \mu\text{M}$ ). In the presence of noncognate alanine, a burst amplitude corresponding to the enzyme concentration used in the reaction is observed for AMP formation (Fig. 4 and Table 3). Significantly, this build-up of AMP within the first turnover of the enzyme further supports the notion that adenylate hydrolysis is primarily an enzyme-catalyzed event.

In the presence of cognate proline and high enzyme concentrations, a burst in AMP formation is also observed (Fig. 4 and

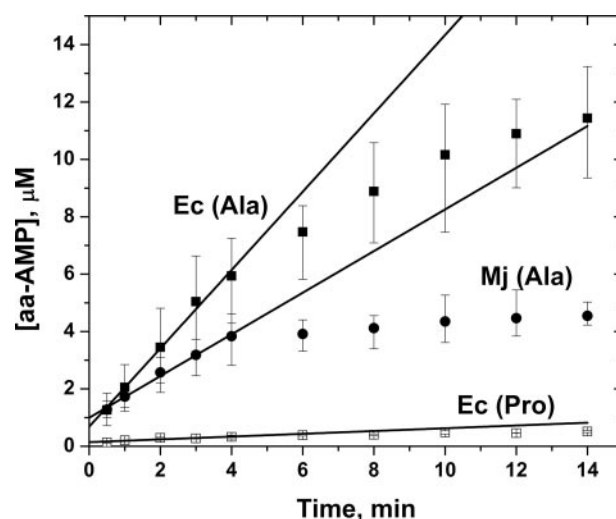


FIGURE 5. Time course of aminoacyl-AMP formation under burst conditions using  $5 \mu\text{M}$  WT Ec ProRS in the presence of alanine (■) and proline (□) and  $5 \mu\text{M}$  Mj ProRS in the presence of alanine (●). All of the reactions were performed in the absence of  $\text{tRNA}^{\text{Pro}}$  as described under "Experimental Procedures."

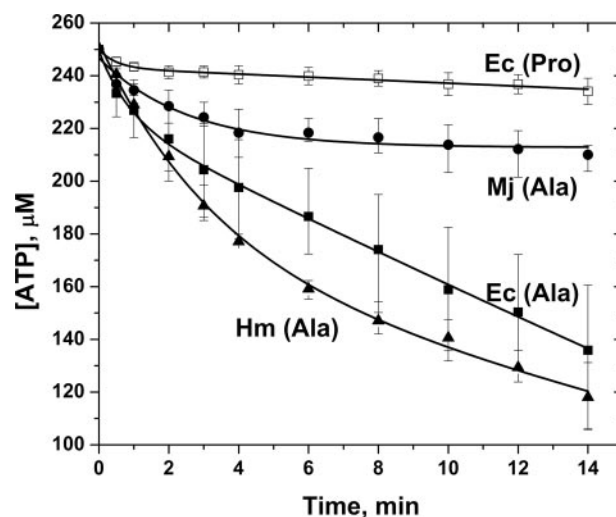


FIGURE 6. Time course of ATP consumption during amino acid activation and hydrolysis of aminoacyl-AMP using  $5 \mu\text{M}$  WT Ec ProRS in the presence of alanine (■) and proline (□),  $5 \mu\text{M}$  Hm ProRS in the presence of alanine (▲), and  $5 \mu\text{M}$  Mj ProRS in the presence of alanine (●). All of the reactions were performed in the absence of  $\text{tRNA}^{\text{Pro}}$  as described under "Experimental Procedures." The lines are drawn to guide the eye.

Table 3), albeit with a significantly slower steady-state rate constant relative to alanine, whereas prolyl-adenylate formation extrapolates to zero at zero time (Fig. 5). Significantly, under these high enzyme conditions, the observation of appreciable AMP formation in the presence of proline indicates that enzyme-catalyzed adenylate hydrolysis can serve as a nonproductive pathway even in the presence of cognate amino acid. However, quantification of the ATP consumed in the presence of alanine and proline revealed that multiple turnovers are observed in the presence of alanine, whereas only a few rounds of hydrolysis are observed in the presence of proline (Fig. 6). The failure to observe AMP formation in the presence of proline at low enzyme concentration (Table 2) underscores the relatively small significance of this pathway for the cognate amino acid.

To probe the site of Ala-AMP hydrolysis by ProRS, we performed similar experiments using Hm and Mj ProRS, which lack the insertion domain capable of post-transfer editing. In the case of Mj ProRS, AMP formation exhibited a burst amplitude corresponding to the total enzyme concentration, with a steady-state rate constant 2-fold lower than that of Ec ProRS (Fig. 4 and Table 3). The steady-state rate of Ala-AMP formation by Mj ProRS was also reduced 2-fold, indicating that, similar to Ec ProRS, this enzyme was 84% efficient in clearing misactivated Ala-AMP (Fig. 5 and Table 3). Monitoring of ATP consumption revealed that unlike Ec ProRS, Mj ProRS only appears to undergo a few turnovers in the presence of noncognate alanine (Fig. 6), which may be due to the assay temperature of 37 °C, which is suboptimal for this thermophilic enzyme.

In the presence of alanine, Hm ProRS also produces multiple turnovers of AMP, but in contrast to Ec and Mj ProRS, no burst amplitude was observed within experimental error (Fig. 4 and Table 3). Additionally, formation of Ala-AMP in the presence of Hm ProRS was not observed, indicating that this synthetase is 100% efficient in clearing misactivated Ala-AMP. Hm ProRS clearly undergoes multiple rounds of hydrolysis, which is evident by monitoring ATP consumption. Because both Mj and Hm ProRS lack a separate post-transfer editing domain, these data suggest that hydrolysis of misactivated amino acids takes place within the aminoacylation active site of the enzyme, consistent with our earlier studies of pre-transfer editing (14).

## DISCUSSION

The presence of multiple editing pathways (Scheme 1) among the AARS highlights the importance of editing mechanisms in the fidelity of protein synthesis (33). Interestingly, the relative contribution of pre- versus post-transfer editing is not a class-conserved feature of the synthetases. For example, the class I editing enzymes IleRS, ValRS, and LeuRS all possess an extra domain, termed connective polypeptide 1 (CP1), at which editing activity is centered (8). Despite the high homology of the domain, the enzymes rely on different editing pathways to maintain fidelity. IleRS is believed to primarily rely on pre-transfer editing of Val-AMP (7). In contrast, ValRS employs post-transfer editing, as evidenced by the accumulation of the Thr-tRNA<sup>Val</sup> intermediate (34). In the case of LeuRS, the dominant editing pathway is species specific. Ec LeuRS has been shown to exhibit only post-transfer editing, whereas the yeast enzyme relies primarily on pre-transfer editing (35). Class I MetRS is capable of catalyzing the cyclization of the homocysteine adenylate to form a thiolactone intermediate, representing a form of pre-transfer editing against this amino acid (36, 37). The class II enzymes ProRS (10–14), AlaRS (38, 39), and PheRS (40–42) all exhibit both pre- and post-transfer editing, whereas ThrRS relies only on post-transfer editing (43, 44), and SerRS performs only pre-transfer editing (9).

Although many synthetases display multiple editing mechanisms, in some cases the dominant pathway is unknown, and the reason for multiple pathways is not well understood. The new results reported herein for Ec ProRS identify specific scenarios wherein both pre- and post-transfer editing play important roles in maintaining the fidelity of the aminoacylation reaction. We show that in the presence of tRNA<sup>Pro</sup>, pre-transfer

editing is not sufficient to prevent the efficient production of Ala-tRNA<sup>Pro</sup> by a post-transfer editing defective ProRS variant, highlighting the dominance of post-transfer editing under these conditions. However, in the absence of tRNA<sup>Pro</sup>, alanine stimulates ATP hydrolysis and AMP formation, indicative of pre-transfer editing. We speculate that in bacteria, pre-transfer adenylate hydrolysis and post-transfer deacylation provide redundant mechanisms to efficiently clear misactivated alanyl-adenylate when the tRNA<sup>Pro</sup> is not present and mischarged Ala-tRNA<sup>Pro</sup> when it is.

Our previous studies on the pre-transfer editing mechanism in the absence of tRNA (14), together with the present studies, highlight the importance of enzyme-catalyzed Ala-AMP hydrolysis in the absence of bound tRNA. Selective release is shown to be a minor pathway ( $\leq 20\%$ ) for clearing aminoacyl-adenylate that dissociates from the active site prior to hydrolysis. In contrast to earlier studies, we performed the present assays in the absence of inorganic PP<sub>i</sub>ase, allowing for the contributions of both the forward and reverse reactions in alanine activation to be observed. The rate for AMP formation in the presence of PP<sub>i</sub>ase ( $0.45 \text{ s}^{-1}$ ) is considerably faster than the rate observed in the absence of PP<sub>i</sub>ase ( $0.034 \text{ s}^{-1}$ ), implying that the reverse reaction is significant in preventing the misactivation of alanine. However, despite this contribution, Ala-AMP is still formed and subsequently hydrolyzed in an enzymatic reaction, highlighting the need for tRNA-independent pre-transfer editing.

Experiments conducted under burst conditions revealed relative rate constants. The amount of AMP extrapolated to time 0 in the presence of alanine by Ec and Mj ProRS corresponds closely to the enzyme concentration used in the assay, indicating that formation of Ala-AMP is faster than its hydrolysis. In addition, the steady-state rate constant of Ala-AMP hydrolysis is significantly faster than hydrolysis of Ala-AMP by water (14, 32) and therefore can be attributed to an enzyme-catalyzed event. In both Ec and Mj systems, the enzyme is not 100% efficient in clearing misactivated Ala-AMP, and slow accumulation of Ala-AMP is observed, suggesting that selective release followed by water hydrolysis plays a minor role in editing. In the presence of cognate amino acid, Ec ProRS undergoes a few rounds of prolyl-adenylate hydrolysis, at which point the enzyme is arrested. Therefore, enzyme-catalyzed prolyl-adenylate hydrolysis, which represents an undesired pathway, is minimized.

Surprisingly, in contrast to Ec and Mj ProRS, Hm ProRS does not exhibit burst kinetics in the presence of alanine while clearly undergoing multiple rounds of hydrolysis. This suggests that in the case of Hm ProRS, formation of Ala-AMP is rate-limiting compared with its hydrolysis. The lack of Ala-AMP accumulation in the presence of the human enzyme also suggests that enzymatic pre-transfer editing is sufficient in this system. In the context of drug design, species-specific mechanisms in amino acid activation and/or editing may, in principal, be exploited in the development of species-selective inhibitors of ProRS.

Examination of the data in Table 3 reveals that all three enzymes exhibit comparable rates of pre-transfer adenylate hydrolysis. Therefore, despite lacking post-transfer editing, Mj

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ProRS and Hm ProRS have not evolved more efficient mechanisms for clearing misactivated Ala-AMP. We previously reported that both Mj and Hm ProRS exhibit selectivity for proline activation relative to alanine that is an order of magnitude greater than that of Ec ProRS. Thus, rather than evolve more efficient editing mechanisms, ProRSs from these species appear to have evolved a more selective active site. Nevertheless, the fact that ProRSs from all three kingdoms exhibit enzyme-catalyzed Ala-AMP hydrolysis supports the relevance of a pre-transfer editing mechanism that *does not* involve translocation to a distant site on the enzyme.

In conclusion, mutational isolation of the pre-transfer editing pathway in Ec ProRS has allowed the relative contributions of both pre- and post-transfer editing of alanine to be assessed. In the absence of tRNA, enzyme-catalyzed adenylate hydrolysis clears misactivated alanine with selective release constituting a minor pathway. However, in the presence of tRNA, a mutant enzyme that is deficient in post-transfer editing efficiently mischarges tRNA<sup>Pro</sup> with alanine. Furthermore, the production of AMP is stoichiometric with the formation of Ala-tRNA<sup>Pro</sup>, indicating that pre-transfer editing is not capable of preventing mischarging under these conditions, highlighting the importance of post-transfer editing. Taken together, these results reveal that both pre- and post-transfer editing work in concert to preserve the fidelity of aminoacylation by bacterial ProRS. In the absence of tRNA, ProRSs from all three kingdoms of life effectively clear misactivated Ala-AMP via enzyme-catalyzed hydrolysis. Because eukaryotic-like ProRSs lack the post-transfer editing domain, these data strongly suggest that pre-transfer editing occurs in the aminoacylation active site.

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