tRNA-independent Pretransfer Editing by Class I Leucyl-tRNA Synthetase

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Aminoacyl-tRNA synthetases catalyze the formation of aminoacyl-tRNA in a two-step reaction starting with amino acid activation followed by aminoacyl group transfer to tRNA. To clear mistakes that occasionally occur, some of these enzymes carry out editing activities, acting on the misactivated amino acid (pretransfer editing) or after the transfer on the tRNA (post-transfer editing). The post-transfer editing pathway of leucyl-tRNA synthetase has been extensively studied by structural and biochemical approaches. Here, we report the finding of a tRNA-independent pretransfer editing pathway in leucyl-tRNA synthetases from Aquifex aeolicus. Using a CP1-mutant defective in its post-transfer editing function, we showed that this new editing pathway is distinct from the post-transfer editing site and may occur at the synthetic catalytic site, as recently proposed for other aminoacyl-tRNA synthetases.

Aminoacyl-tRNA synthetases catalyze the esterification reaction between an amino acid and its cognate tRNA (1, 2). These enzymes are specific for both amino acid and tRNA substrates, ensuring the high fidelity required by translation. Mistakes caused by these synthetases can result in genetic code ambiguity and disruption of cellular functions (3, 4). The aminoacylation of tRNA is a two-step reaction: (a) activation of amino acids with ATP by forming aminoacyl adenylates (AA-AMP)3 followed by (b) transfer of the aminoacyl residues from the aminoacyl adenylates to the cognate tRNA substrate (2). The accuracy of aminoacylation depends on both the specific recognition of amino acids during their activation (“coarse sieve”) and the editing that corrects errors at either the aminoacyl adenylate level (pretransfer editing) or the aminoacyl-tRNA level (post-transfer editing or “fine sieve”) (5). The 20 aminoacyl-tRNA synthetases can be divided into two classes of 10 enzymes on the basis of conserved sequences and characteristic structural motifs (6). Leucyl-tRNA synthetase (LeuRS), isoleucyl-tRNA synthetase (IleRS), and valyl-tRNA synthetase belong to class Ia. LeuRS, IleRS, and valyl-tRNA synthetase develop a separated editing domain (also called the CP1 domain) that is appended to the aminoacylation active site to distinguish structurally similar Leu, Ile, and Val and to eliminate misacylated products (7–9). Editing activity has also been demonstrated in a number of class II synthetases, including alanyltRNA synthetase (10, 11), threonyl-tRNA synthetase (12), prolyl-tRNA synthetase (13, 14), seryl-tRNA synthetase (15), and phenylalanyl-tRNA synthetase (16).

The mechanism of post-transfer editing is relatively clear. Based on structural and biochemical data, a model has been proposed wherein the flexible 3’-end of a misacylated tRNA is translocated from the aminoacylation active site to the hydrolytic editing site (8, 21–24). In contrast, the mechanism of pre-transfer editing is less well understood. Fluorescence-based assays and mutational analysis, together with x-ray crystallography studies showing that both pre- and post-transfer editing substrate analogs bind in overlapping sites in the CP1 domain of LeuRS and IleRS, have led to the proposal that misactivated amino acids are translocated from the catalytic site to the editing domain in a tRNA-dependent manner (8, 17–19). A “post-initiated pretransfer editing” model has been postulated for IleRS. In this model, an initial post-transfer editing step triggers a conformational change to an editing active conformation that can perform pretransfer editing in the CP1 domain (20). However, based on x-ray crystallography data, the aminoacylation and editing active sites are separated by ~30 Å, and no confined passageway between active sites serving to prevent dissociation of AA-AMP from the surface of the enzyme during translocation is apparent. Thus, the mechanism of this translocation remains unclear. On the other hand, a recent study of class I glutaminyl-tRNA synthetase, which normally lacks editing function and a spatially separate editing domain, has provided evidence for a tRNA-dependent pretransfer editing-like reaction occurring in the aminoacylation active site (21).

Another intriguing feature of pretransfer editing lies in the tRNA dependence of the reaction. It is well accepted that the pretransfer editing of IleRS, valyl-tRNA synthetase, and LeuRS is tRNA-dependent, consistent with the hypothesis that tRNA plays a role in the translocation of AA-AMP from the catalytic
site to the editing domain. However, a recent study has revealed that, although it carries an extra editing domain, the class II prolyl-tRNA synthetase catalyzes Ala-AMP hydrolysis predominantly into the synthetic active site in a tRNA-independent way, whereas the “selective release” of the noncognate adenylate from the active site into solution constitutes only a minor pathway (22).

The recently designed experiments based on TLC analysis (21) led us to further investigate the pretransfer editing function of class I LeuRS, with emphasis on tRNA independence and the synthetic catalytic site. LeuRSs from the bacterium *Aquifex aeolicus* (AaLeuRS) was used in the study. In addition, the editing properties of post-transfer editing-deficient mutants of AaLeuRS were also studied.

**EXPERIMENTAL PROCEDURES**

**Preparation of Enzymes and RNA Substrates—**AaLeuRS and its mutant D373A were overproduced in *Escherichia coli* as His-tagged proteins and purified by Ni²⁺-nitrilotriacetic acid chromatography, as previously described (24). *Aa*rRNA²⁴ was purified from an *E. coli* overproduction strain (25, 26); the tRNA charging level was 1400 pmol/*Aa*σo*. *Aa*rRNA²⁴ was prepared using AaLeuRS D373A.

**ATP/PP₆ Exchange, tRNA Charging, and Decacylation—**The ATP-PP₆ exchange (60 °C) and isoleucylation (37 °C) assays of AaLeuRS were performed as previously described (24). ATP-PP₆ exchange kinetics of enzymes were determined in the presence of 0.02–0.2 mM Leu and 0.25–64 mM Nva, Ile, or Met. The hydrolytic editing assays of AaLeuRS were performed at 37 °C (24).

**ATP Consumption—**Assays for AaLeuRSs were carried out in a reaction mixture containing 100 mM Tris-HCl (pH 7.8), 30 mM KCl, 12 mM MgCl₂, 5 mM dithiothreitol, 5 units/ml pyrophosphatase, 3 mM [γ-³²P]ATP (20–30 cpm/pmol), 15 mM amino acids, and 1 μM LeuRS with or without 5 μM cognate tRNALeu at 60 °C. Aliquots (1 μl) of the reaction mixture were removed at appropriate time intervals and quenched in 350 μl of a mixture containing 10 mM tetrasodium pyrophosphate, 7% HClO₄, and 6% activated charcoal. After centrifugation, the amount of inorganic phosphate ³²P in 50 μl of supernatant was quantified by scintillation counting. The background rate in the ATP consumption assay in the absence of amino acid was subtracted.

**AMP Formation—**For AaLeuRS, AMP formation was measured in reaction mixtures containing 100 mM Tris-HCl (pH 7.8), 30 mM KCl, 12 mM MgCl₂, 5 mM dithiothreitol, 5 units/ml pyrophosphatase, 3 mM ATP, 20 mM [α-³²P]ATP (3000 Ci/mmol; Amersham Biosciences), and 15 mM Leu or 15 mM Nva or 30 mM Ile, with or without 5 μM *Aa*rRNA²⁴. Reactions were incubated at 60 °C and initiated by the addition of 1 μM AaLeuRS or its D373A mutant. Aliquots (1.5 μl) were quenched in 6 μl of 200 mM sodium acetate (pH 5.0). Quenched aliquots (1.0 μl each) were spotted in duplicate on polyethyleneimine-cellulose plates (Merck) prewashed with water. Separation of AA-³²PAMP, [³²P]AMP, and [³²P]AMP was performed by developing TLC plates in 0.1 M ammonium acetate and 5% acetic acid (21, 22). The plates were visualized by phosphorimaging, and data were analyzed using ImageQuant version 5.2 software (GE Healthcare). The positions corresponding to the elution of free AMP were confirmed by using commercially available AMP (Sigma), and the positions corresponding to AA-³²PAMP and AA-[³²P]AMP were compared with the gray density of a known [³²P]ATP concentration. Rate constants were obtained from graphs of AA-[³²P]AMP and [³²P]AMP formation plotted against time. Because Leu-AMP is the cognate product, its binding to LeuRS is very tight, and we could not detect it by the TLC procedure described above without using a modified quenching buffer containing 0.5% SDS, 250 mM ATP, 25 mM EDTA, 200 mM NaAc, pH 4.7. Under these denaturing conditions, the released Leu-AMP could be detected (Fig. S2).

**Nonenzymatic Hydrolysis of Adenylates—**The rate of nonenzymatic hydrolysis of adenylate was measured by a chase experiment wherein a large excess of unlabeled ATP was added to the reaction mixtures following the initiation of Nva-AMP synthesis (21). The excess of unlabeled ATP induces the release of the noncognate aminoacyl-adenylate from the active site into solution, where its spontaneous hydrolysis is monitored on TLC plates. A solution of 1 μM AaLeuRS was first incubated with 15 mM Nva, 100 mM ATP, and 0.25 μM [α-³²P]ATP for 12 min at 60 °C to prepare Nva-[³²P]AMP. Then unlabeled ATP (120 mM, equal to a 1200-fold molar excess of unlabeled ATP) was added, and the hydrolysis activity was quenched at various time points (0.5–10 min) by mixing 2 μl of the reaction mixture with 6 μl of 200 mM sodium acetate (pH 5.0), 0.1% SDS. Separation of Nva-[³²P]AMP and [³²P]AMP by TLC was then performed, and reactions were quantified as described above. The rate constants for the nonenzymatic hydrolysis reactions were obtained by plotting the concentration of Nva-[³²P]AMP against time and fitting the data to a first-order decay curve.

**RESULTS**

**AaLeuRS Misactivates and Edits Norvaline—**In cognate aminoacylation, the formation of one aminoacyl-tRNA molecule leads to the transient synthesis of one aminoacyl-adenylate molecule and the consumption of a single ATP molecule. However, when a noncognate amino acid is activated and then hydrolyzed at the aminoacyl-adenylate or later aminoacyltRNA level, one molecule of ATP is wasted (27, 28). Therefore, the extra consumption of ATP during enzyme catalysis is characteristic of editing events.

In this study, we measured and compared ATP consumption of the bacterial AaLeuRS for Leu and its analogs Nva, Ile, and Met. These amino acids triggered ATP consumption at different levels in the order Nva ≫ Met > Ile > Leu in the presence of tRNA²⁴. In the absence of tRNA, the order was Nva ≫ Ile > Met > Leu, and the consumption was clearly lower (Fig. 1). As expected, AaLeuRS consumed negligible ATP in the presence of Leu, the cognate substrate, because there was no waste of ATP due to editing. Nva triggered the strongest ATP consumption, suggesting a very active editing process. In the absence of tRNA, ATP consumption was reduced but not abolished, sug-
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**FIGURE 1.** Editing of various amino acids by AaLeuRS. **A**, using 1 μM AaLeuRS, ATP consumption in the presence of 15 mM Leu, Nva, Ile, or Met with 5 μM antRNA<sub>Leu</sub>, same as A but without tRNA. The inset shows the structures of amino acids involved in this section. Data were the average of three independent assays.

**TABLE 1**
Kinetic constants of AaLeuRS in amino acid activation reaction at 60 °C

| Amino acid | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | $k_{cat}/K_m$ (relative) |
|------------|-------|----------|---------------|--------------------------|
| Leu        | 2.8 ± 0.4 | 26.3 ± 3.3 | 9.4 ± 1.3 | 1 ± 0.1 |
| Nva        | 96 ± 12  | 12.6 ± 1.4  | 0.13 ± 0.013 | 1.4 × 10<sup>-2</sup> |
| Ile        | 698 ± 109 | 1.9 ± 0.3 | 2.7 × 10<sup>-3</sup> | 2.9 × 10<sup>-4</sup> |
| Met        | 983 ± 139 | 3.2 ± 0.4 | 3.3 × 10<sup>-3</sup> | 3.5 × 10<sup>-4</sup> |

**TABLE 2**
Steady-state rate constants of AaLeuRS and its D373A mutant in AMP and AA-AMP synthesis at 60 °C

| LeuRS | Amino acid | tRNA | AMP formation $k_{obs}$ | AA-AMP formation $k_{obs}$ |
|-------|------------|------|-------------------------|---------------------------|
| WT    | Leu        |      | (3.4 ± 0.6) × 10<sup>-2</sup> | ND*                        |
| WT    | Leu        |      | (8.8 ± 0.1) × 10<sup>-3</sup> | ND                        |
| WT    | Ile        |      | (8.2 ± 0.1) × 10<sup>-2</sup> | ND                        |
| WT    | Ile        |      | (1.6 ± 0.1) × 10<sup>-2</sup> | ND                        |
| WT    | Nva        |      | 1.5 ± 0.2                | ND                        |
| WT    | Nva        |      | (9.8 ± 1.5) × 10<sup>-2</sup> | (6.6 ± 0.8) × 10<sup>-3</sup> |
| D373A | Nva        |      | (2.9 ± 0.3) × 10<sup>-1</sup> | (5.0 ± 0.6) × 10<sup>-3</sup> |
| D373A | Nva        |      | (9.8 ± 1.3) × 10<sup>-2</sup> | (6.3 ± 0.9) × 10<sup>-3</sup> |

* ND, not detected.

suggesting the existence of a tRNA-independent editing mechanism in addition to tRNA-dependent editing.

In addition, we measured Nva activation by the ATP-PP<sub>i</sub> exchange assay and compared it with the other amino acids. Compared with leucine, Nva was efficiently activated. The values of $k_{cat}$ decreased by half, and $K_m$ increased 35-fold for Nva, which indicated that the enzyme needed more substrate to reach half-saturation; the global effect corresponded to a 70-fold loss in catalytic efficiency (Table 1). AaLeuRS also activated Ile and Met but with a 40–50-fold lower efficiency than it did with Nva (Table 1). Taken together, these results showed a strong correlation between amino acid activation efficiency and ATP consumption levels. Nva, the most efficiently activated analog, consumed the largest amount of ATP. A significant level of ATP consumption was also measured in the absence of tRNA, indicating that proofreading occurs at the aminoacyl-adenylate level independently of the presence of tRNA.

**AaLeuRS Possesses an Efficient tRNA-dependent Editing Pathway**—In the previous assay, we showed that AaLeuRS activated the noncognate amino acids Nva, Ile, and Met and consumed an extra amount of ATP to probably edit these noncognate products. We applied a TLC-based assay to simultaneously follow $[^32]P$AMP and aminocycl-[^32]P]adenylate formation in the presence of noncognate amino acids and tRNA. The same assay was performed without tRNA in order to investigate the tRNA-independent editing step. Our data showed that Nva stimulated much more $[^32]P$AMP formation than Ile or Leu, in accordance with the ATP consumption results. Control reactions showed that there was very little AMP formation in the absence of enzyme or amino acid (Fig. S1). In the presence of tRNA, the rate of AMP formation in the presence of 15 mM Nva was 18-fold ($k_{obs}$ ratio 1.5:0.082) greater than the rate of AMP formation in the presence of 30 mM Ile and 44-fold ($k_{obs}$ ratio 1.5:0.034) greater than that in the presence of 15 mM Leu (Table 2, Fig. 2, and Fig. S3). A significant amount of Nva-$[^32]P$AMP was also detected, but its formation rate was only 1/500 ($k_{obs}$ ratio 0.0031:1.5) of the rate of $[^32]P$AMP formation.

**An Additional Minor tRNA-independent Editing Pathway to Edit Norvaline**—In the absence of tRNA, the AMP formation was considerably reduced (Table 2, Fig. 2, and Figs. S2 and S3). The rate of AMP formation in the presence of 15 mM Nva was only 1/5 times ($k_{obs}$ ratio 0.098:1.5) the corresponding rate in the presence of tRNA, whereas those in the presence of Leu and Ile were very close to the background level (Table 2, Fig. 2, and Fig. S3). Therefore, these data suggested that tRNA-dependent editing was the main pathway used by AaLeuRS to clear the misactivated compounds. However, the data also showed the existence of an additional process, weaker in efficiency, which did not depend on the tRNA. In addition, the data showed that in the absence of tRNA, the rate of Nva-$[^32]P$AMP formation was increased 2-fold, and its concentration exceeded the enzyme concentration when the reaction was carried out for 12 min (Table 2 and Fig. 2). This might indicate that a fraction of this compound was released into solution instead of being edited on the enzyme by a tRNA-independent process. Once released into solution, Nva-$[^32]P$AMP might undergo noncatalytic hydrolysis. In order to measure the rate of this later reaction, we performed a cold chase experiment. The rate of Nva-$[^32]P$AMP hydrolysis was insensitive to the concentration of ATP in this concentration range and yielded a $k_{obs}$ of 0.0004 s<sup>-1</sup> (Fig. 3). 245-fold lower than the $k_{obs}$ determined for AMP formation (0.098 s<sup>-1</sup> in Table 2), which takes into account both catalytic and noncatalytic AMP formation (Fig. 3 and Table 2). This showed that the rate of hydrolysis of Nva-$[^32]P$AMP after its release into solution was negligible compared with the total hydrolysis rate measured in the presence of Nva. Thus, the rate of AMP formation was not driven by nonenzymatic adenylate hydrolysis but by another faster step.

**tRNA-independent Editing Does Not Occur in the CP1 Editing Domain**—To explore the role of the CP1 domain in the tRNA-independent editing of AaLeuRS, we inactivated the CP1
domain by mutating a conserved aspartic acid of AaLeuRS to alanine (D373A). This residue has been shown to be crucial for maintaining the proper orientation of both pre- and post-transfer editing substrates into the CP1 domain (29), and its mutation in E. coli and yeast LeuRS abolishes the editing activity (30).

When introduced into AaLeuRS, the D373A mutation abolished the post-transfer editing of the mischarged Ile-tRNALeu (Fig. 4). The D373A mutation also reduced to \( \frac{1}{5} \) \( k_{\text{obs}} \) ratio \( 0.29:1.5 \) the rate of AMP formation measured in the presence of Nva in the TLC assay (Figs. 1 and 4 and Table 2). This was expected, according to the loss of post-transfer editing monitored in the deacylation assay. However, the \( k_{\text{obs}} \) value of the mutant for AMP formation with tRNA was 3-fold \( (k_{\text{obs}} \text{ ratio } 0.29:0.098) \) higher than that of the native enzyme monitored without tRNA (Table 2). This showed that a mutant exhibiting an inactive CP1 domain, in the presence of tRNA, consumed more ATP than the native LeuRS in the absence of tRNA. If we assume that the CP1 domain was completely inactivated, this would mean that tRNA\(^{\text{Leu}}\) was also involved in an editing process not located in the CP1 domain.

The \( k_{\text{obs}} \) of the AaLeuRS-D373A mutant with tRNA in Nva-[\( ^{32} \)P]AMP formation was 0.005 s\(^{-1} \) and increased slightly more than that of the native AaLeuRS (0.0031 s\(^{-1} \)) (Fig. 5 and Table 2). This indicated that in the absence of efficient post-transfer editing and with a constant synthesis rate, there was an increase in the steady-state rate formation of Nva-[\( ^{32} \)P]AMP. As a consequence, the accumulation of noncognate adenylates by mutant D373A induced a weak but significant mischarging of tRNALeu, as shown in the aminoacylation assay (Fig. 4). This also indicated that the remaining editing activity was not dependent on the CP1 domain and was not sufficient to prevent the misaminoacylation of tRNALeu.

More interestingly, in the absence of tRNA, when only the tRNA-independent editing was monitored, the AMP formation was identical to that measured with the native AaLeuRS (both \( k_{\text{obs}} \) 0.098 s\(^{-1} \)) corresponding to 7% (0.098/1.5) of the total editing activity of the native enzyme in the presence of tRNA, however, 93% of AMP formation resulted from a tRNA-dependent reaction (Table 2). Although it showed no detectable post-transfer editing activity (Fig. 3), mutant D373A in the presence of tRNA displayed an additional 12.8% (0.29–0.098/1.5) AMP formation activity as compared with the assay without tRNA (Table 2).

This suggested that tRNA\(^{\text{Leu}}\) might be involved in an editing process not located in the CP1 domain or, alternatively, that the D373A mutation did not totally inactivate the CP1 editing site.

FIGURE 2. TLC of a reaction time course showing the separation of [\( ^{32} \)P]AMP and Nva-[\( ^{32} \)P]AMP. Reactions were carried out with AaLeuRS in the presence (+tRNA) or absence of tRNA (−tRNA). Each graphic represents the data shown in the panel above. The region of Nva-AMP produced by AaLeuRS is darkened and marked by a shaded box for better visibility.
DISCUSSION

Norvaline Editing May Be Essential for Maintaining Translation Fidelity—A number of previous studies of the LeuRS editing function have used Ile as a noncognate amino acid substrate. Here, we showed that AaLeuRS activated Ile with a rate 3500-fold lower than that of the cognate Leu. Such a low misactivation rate is comparable with the general error level in protein synthesis, 1:3000 (31), suggesting that the proofreading of Ile-tRNALeu is not critical for the cell. In contrast, we showed here that Nva was activated by AaLeuRS with an efficiency only 70-fold lower than that of Leu. Since Nva differs from Leu by a single methyl group, its smaller size and structural similarity with Leu make it less distinguishable by the coarse sieve of the synthetic site of LeuRS (5). Nva is naturally found in vivo. Norvaline has been reported in an antifungal peptide produced by Bacillus subtilis (32) and as an Ile fermentation product (33). Nva has also been reported to be a potential by-product of the Leu biosynthetic pathway in E. coli (23). Since there is no clear mechanism to utilize Nva, it accumulates, and the ratio of free Nva/Leu rises. This leads to increasing mischarge of leucine-specific tRNAs with Nva, resulting in some conditions in the detectable incorporation of Nva into proteins (23). Such a high misactivation rate of Nva by LeuRS may threaten the fidelity of translation, and we hypothesize that the enzyme may have evolved some editing mechanism against Nva.

Post-transfer Editing Is the Major Pathway for Nva Editing—To investigate the editing reactions of AaLeuRS, we performed two experiments. (a) First, we monitored AMP and Nva-AMP formation in the presence or absence of tRNA in order to separate the contribution of the tRNA-dependent editing from tRNA-independent editing. Because 93% of AMP formation resulted from a tRNA-dependent reaction and only 7% from a tRNA-independent reaction, AaLeuRS preferentially uses the fine sieve of the post-transfer editing reaction as valyl-tRNA synthetase, another class Ia synthetase (34, 35), and in contrast to E. coli IleRS, which proceeds 70–90% through the pretransfer editing pathway (28). (b) Second, we repeated the same experiment on a LeuRS mutant carrying an inactive CP1 domain in order to measure the contribution of the CP1 domain to the formation of AMP. To inactivate the CP1 domain, residue Asp-373 was mutated to Ala in order to mimic the corresponding E. coli and Saccharomyces cerevisiae LeuRS mutants that are deficient in editing (29). According to the x-ray structure of TtLeuRS, this conserved acidic residue has been shown to interact with both post-transfer and pretransfer analogs (29). Our results clearly showed that the tRNA-independent editing activity was not decreased by the D373A mutation, suggesting that this activity was not confined to the CP1 domain but resided most likely at the synthetic site, as observed for other editing aminoacyl-tRNA synthetases lacking separated editing domains (15, 21).
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**Kinetic Proofreading and Pretransfer Editing to Achieve LeuRS Specificity**—The TLC assay revealed significant Nva-AMP accumulation when the editing reaction was hampered by the absence of tRNA or when the inactivated D373A mutant was used. Under those conditions, Nva-AMP formed and accumulated if the pretransfer editing activity was not efficient enough to clear the noncognate adenylate molecules.

In addition to post-transfer editing (Fig. 7, route 4) (34, 36), hydrolytic editing of errors of misactivation of amino acids can take place by three other routes, as shown in Fig. 7: (a) via route 1, the dissociation of the complex to give the free aminocyl adenylate complex, which hydrolyzes in solution (“kinetic proofreading”) (27) or (b) via routes 2 and 3, the hydrolysis of aminocyl-adenylate when bound to the enzyme. We have evidence from this study that these pathways could be used to some extent.

With regard to kinetic proofreading (route 1), it has been pointed out previously that the rate constant for the dissociation of the cognate aminocyl-adenylate from its enzyme is in general low, and consequently, only very dissimilar noncognate complexes should dissociate sufficiently rapidly to compete significantly with transfer (37). Here, we showed that by a tRNA-independent process, AMP was formed with a $k_{\text{obs}}$ of 0.098 s$^{-1}$. The dissociation and accumulation (or formation) of Nva-AMP into the reaction mixture was also observed with a $k_{\text{obs}}$ of 0.0066 s$^{-1}$ (Table 2). In contrast, under the same conditions, the release of Leu-AMP was not observed after the cognate activation reaction, suggesting that the stability of the cognate adenylate on the enzyme was much higher than that of Nva-AMP. To observe Leu-AMP release, protein-denaturing conditions were required, confirming that the cognate adenylate is tightly bound. These observations showed that preferential or selective dissociation occurred with Nva-AMP compared with Leu-AMP. However, this phenomenon cannot contribute significantly to the global AMP formation, since our data indicate that Nva-AMP accumulation rate was low despite good stability of Nva-AMP in solution, the spontaneous nonenzymatic hydrolysis rates being 0.0004 s$^{-1}$ at 60 °C and 0.0001 s$^{-1}$ at 30 °C, much lower that the tRNA-independent AMP formation rate (0.098 and 0.14 s$^{-1}$ for AaLeuRS). These data strongly suggested that the nonenzymatic degradation of noncognate adenylates did not play a significant role in the global editing reaction. This also indicated that there is another rate-determining step in the pretransfer tRNA-independent editing mechanism. The rate-determining step might be, for example, another editing step catalyzed by the enzyme at the pretransfer level (e.g. route 2). Such editing reactions have recently been described for glutaminyl-tRNA synthetase and seryl-tRNA synthetase (15, 21). Although conceptually described in the 1960s (38), the enzyme-catalyzed pretransfer editing (route 2) has proven to be more difficult to elucidate, essentially because of the inability to directly assess its pathway. Evidence in favor of pretransfer editing was obtained by detailed kinetic investigations performed on IleRS (28). Pretransfer editing includes several mechanisms that remove the adenylate before the aminoclaylation catalysis, as shown in Fig. 7. It can occur at the synthetic site in the absence of tRNA (Fig. 7, route 2) or in the presence of cognate tRNA (Fig. 7, route 3) (28) and, alternatively, at a distinct hydrolytic site, such as the CP1 editing site. In that case, the noncognate adenylate has to be transferred to the editing site in a tRNA-dependent manner, and this would require a complex mechanism (19, 20) and structural rearrangements (39). As suggested before, the rate-determining step for the 12.8% residual AMP formation activity observed with the D373A mutant might also be the rate of a tRNA-dependent pretransfer editing reaction. Although we did not demonstrate its existence, we cannot exclude the existence of such activity in AaLeuRS. Such hydrolytic activity, occurring at the synthetic site, was proposed for glutaminyl-tRNA synthetase (21) and seryl-tRNA synthetase (15), two other aminocyl-tRNA synthetases lacking editing sites.

In summary, our findings suggested that AaLeuRS follows the “double sieve” theory for rejection of noncognate amino acids (5). The coarse sieve mechanism rejects most of the noncognate amino acids during the activation step. As a consequence, Ile and Met are poorly activated, whereas Nva escapes partially to this sieve. The second sieve, or fine sieve, includes...
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the post-transfer editing activity, which is the major editing pathway, and one additional minor pretransfer editing activity, which does not depend on tRNA. It has been suggested that pretransfer editing activities that still persist in contemporary enzymes are remnants of the initial editing activity. They were operating as crude filters to generate only a small increase in the overall fidelity (21). During evolution, aminoacyl-tRNA synthetases have improved the proofreading mechanism by adding a more sophisticated post-transfer editing mechanism. At the same time, they have evolved separate editing domains. For AaLeuRS, further investigations to locate the exact active site for the tRNA-dependent pretransfer editing pathway are currently under way by constructing and analyzing more editing-deficient mutants.

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