Leucyl-tRNA synthetase editing domain functions as a molecular rheostat to control codon ambiguity in *Mycoplasma* pathogens

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Mycoplasma **leucyl-tRNA synthetases (LeuRSs) have been identified in which the connective polypeptide 1 (CP1) amino acid editing domain that clears mischarged tRNAs are missing (*Mycoplasma mobile*) or highly degenerate (*Mycoplasma synoviae*). Thus, these enzymes rely on a clearance pathway called pretransfer editing, which hydrolyzes misactivated aminoacyl-adenylate intermediate via a nebulous mechanism that has been controversial for decades. Even as the sole fidelity pathway for clearing amino acid selection errors in the pathogenic *M. mobile*, pretransfer editing is not robust enough to completely block mischarging of $tRNA^{Leu}$, resulting in codon ambiguity and statistical proteins. A high-resolution X-ray crystal structure shows that *M. mobile* LeuRS structurally overlaps with other LeuRS cores. However, when CP1 domains from different aminoacyl-tRNA synthetases and origins were fused to this common LeuRS core, surprisingly, pretransfer editing was enhanced. It is hypothesized that the CP1 domain evolved as a molecular rheostat to balance multiple functions. These include distal control of specificity and enzyme activity in the ancient canonical core, as well as providing a separate hydrolytic active site for clearing mischarged tRNA.**

protein evolution | quality control | statistical proteins | aminoacylation | translation

Aminoacyl-tRNA synthetases (aaRSs) catalyze the first step of protein synthesis. Each aaRS covalently attaches amino acid to its cognate tRNA to set the genetic code (1, 2). Faithful translation in modern cells imposes a great challenge for aaRSs as they select cognate substrate amino acids from a pool of structurally similar amino acids (1, 2). As such, about half of the 20 aaRSs have developed editing mechanisms to clear mischarged tRNA products before the noncognate amino acids can be incorporated into newly synthesized proteins at the ribosome.

Robust editing to achieve proteome fidelity has been commonly thought to be essential to the cell (1, 2). Indeed, fidelity can be critical for microbe and mammalian cell viability (3–5), as well as the prevention of neurological disease (5). However, in host-dependent pathogens, we and others have identified aaRS-dependent mechanisms that foster translational infidelity resulting in statistical proteins (6, 7). We hypothesize that some organisms have adapted to promote or tolerate threshold levels of statistical mistranslations. In these cases, multiple aaRS mechanisms that influence fidelity of the aminoacylation reaction can be affected through the evolution process.

All aaRS aminoacylation reactions (Eqs. **1** and **2**) proceed in two steps with aminoacyl-adenylate as the intermediate:

$$\mathrm{ATP} + \mathrm{amino\ acid\ (aa)} \rightleftharpoons \mathrm{aa\text{-}AMP} + \mathrm{PP_i} \qquad [1]$$

$$\mathrm{aa\text{-}AMP} + \mathrm{tRNA}^{aa} \rightarrow \mathrm{aa\text{-}tRNA}^{aa} + \mathrm{AMP}. \qquad [2]$$

Editing reactions have evolved to clear mistakes at both steps of the aaRS catalyzed reaction (8). In the posttransfer editing pathway (Eq. **3**), mischarged tRNA is hydrolyzed in an aaRS domain that is distinct from the synthetic aminoacylation domain (9–16). In addition, editing of mischarged tRNA can occur in *trans* by independent proteins that function as tRNA-specific deacylases (17, 18):

$$\mathrm{aa_x\text{-}tRNA}^{aa} \rightarrow \mathrm{aa_x} + \mathrm{tRNA}^{aa}. \qquad [3]$$

The origin of pretransfer editing (Eq. **4**) that hydrolyzes the adenylate intermediate has been much more controversial:

$$\mathrm{aa_x\text{-}AMP} \rightarrow \mathrm{aa_x} + \mathrm{AMP}. \qquad [4]$$

The editing domain has been proposed to be critical to pretransfer editing (19), in addition to its contribution to the posttransfer editing pathway. Both tRNA-dependent (20–23) and tRNA-independent (22, 24) pretransfer editing activities have been reported. Also, clearance of the adenylate by selective ejection from the enzyme's active site into the aqueous environment has been suggested (24). It is possible that these diverse fidelity mechanisms could be used selectively or combinatorially by different aaRSs to clear noncognate products (8, 25).

In leucyl- (LeuRS), isoleucyl- (IleRS), and valyl-tRNA synthetases (ValRS), the hydrolytic active sites of posttransfer editing have been mapped to their homologous connective polypeptide 1 (CP1) domains (9, 26). The CP1 domain is inserted into the Rossmann fold that comprises the synthetic aminoacylation site via two β-strands (27). In the tRNA-bound editing complex, the LeuRS CP1 domain is extended away from the canonical aminoacylation core (28). However, in the aminoacylation complex that was recently solved for *Escherichia coli* LeuRS, the CP1 descends upon the aminoacylation active site to interact directly and indirectly with the 3′ end of the tRNA (29).

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 3ZIU).

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In LeuRS from the *Mycoplasma mobile* pathogen, this otherwise universally conserved CP1 domain is completely missing (6). Hence, *M. mobile* LeuRS produces tRNA mischarged with noncognate structurally related aliphatic amino acids, resulting in statistical proteins (6). Related LeuRSs from *Mycoplasma synoviae* and *Mycoplasma agalactiae*, which are poultry and sheep pathogens, respectively, have CP1 domains that are highly degenerate and lack any signature sequences that comprise the conventional hydrolytic active site (6) (Fig. S1).

Herein, we present a high-resolution X-ray crystal structure demonstrating that *M. mobile* LeuRS overlaps with the canonical structures of other LeuRSs. Using this *M. mobile* LeuRS core as a scaffold, we created a series of LeuRS hybrids with CP1 domains from different origins as well as from IleRS and ValRS (30). Remarkably, we determined that the addition of the CP1 domain enhances pretransfer editing activity and fidelity for each of these diverse hybrid models. This contrasts with deletion of CP1 editing domains from *E. coli* and *Saccharomyces cerevisiae* mitochondria LeuRSs, which activated a latent tRNA-dependent pretransfer editing activity that suppresses tRNA mischarging (21). We hypothesize, then, that the CP1 domain has evolved to function as a molecular rheostat to enhance fidelity by idiosyncratically potentiating substrate specificity (30) while balancing hydrolytic mechanisms that are associated with either the aminoacylation core or the separate editing domain.

Results

***M. synoviae* LeuRS Naturally Produces Mischarged tRNA^{Leu}.** Hydrolytic CP1 editing domains are ubiquitously conserved in LeuRS, IleRS, and ValRS across all three domains of life (27). Previously, we identified three unique cases of *Mycoplasma* LeuRSs that had altered or missing CP1 domains (6). The *M. mobile* CP1 domain is completely absent, resulting in statistical substitutions in the proteome. *M. synoviae* and *M. agalactiae* contained highly degenerated CP1 domains that we hypothesized also lacked posttransfer editing (Fig. S1).

To test the mischarging activity of *M. synoviae* LeuRS, the gene was synthesized using optimized codon use frequencies for expression in *E. coli* and also to convert triplet TGA stop codons (which are used to encode tryptophan in *M. synoviae*) to TGG. The monomeric LeuRS was purified by affinity chromatography via an N-terminal six-histidine tag. The enzyme robustly aminoacylated in vitro transcribed *M. mobile* tRNA^{Leu} (Fig. 1*A*). As we predicted, *M. synoviae* LeuRS displayed significant mischarging activity (Fig. 1*B*), similar to *M. mobile* LeuRS, which is missing its CP1 domain (6). Likewise, the degenerated CP1 domain failed to deacylate mischarged Ile-tRNA^{Leu} (Fig. 1*C*). We hypothesize that *M. synoviae* LeuRS represents an intermediate evolutionary step on a path to the elimination of the CP1 domain as found in *M. mobile* LeuRS.

***Mycoplasma* LeuRSs Maintain Weak Pretransfer Editing Activities.** *E. coli* LeuRS harbors a tRNA-dependent pretransfer editing activity that is unmasked when its CP1 domain is deleted (21). We also found that we could activate pretransfer editing in *E. coli* LeuRS by incorporating an unchargeable 2′-deoxyadenosine tRNA^{Leu} (2′dA-tRNA) (21). Thus, we hypothesized that the pathway to amino acid editing can shift between pre- and post-transfer editing mechanisms that are inherent to the enzyme (8, 25). Even though *M. mobile* has statistical substitutions at leucine codons, we sought to determine whether the pretransfer editing pathway was activated in this naturally occurring protein where the CP1 domain is eliminated. Likewise, we have also tested pretransfer editing in *M. synoviae* LeuRS where the CP1 domain was present but highly degenerate and yields mischarged tRNAs.

We incorporated unchargeable tRNA analogs that contained a 2′dA-tRNA or dideoxyadenosine-tRNA (ddA-tRNA) at the 3′ acceptor stem end into amino acid-dependent ATP hydrolysis assays. Because 2′dA-tRNA and ddA-tRNA are missing the functional 2′ hydroxyl group that is necessary for aminoacylation to occur, AMP formation in these reactions would reflect cycles of amino acid activation and hydrolysis that are representative of pretransfer editing (31, 32).

The 2′dA-tRNA clearly stimulated AMP production by *M. mobile* LeuRS (Fig. 2*A*) and *M. synoviae* LeuRS (Fig. S2*A*) in the presence of isoleucine, but not the cognate leucine substrate (Fig. 2*B* and Fig. S2*B*). We also determined that this pretransfer editing activity cleared isoleucyl-adenylate when 2′dA-tRNA or ddA-tRNA was added (Fig. 2*C* and Fig. S2*C*). In comparison, leucyl-adenylate was stable when *Mycoplasma* LeuRS and a modified tRNA was present (Fig. 2*D* and Fig. S2*D*). Thus, the synthetic core of *Mycoplasma* LeuRS harbors a bona fide tRNA-dependent pretransfer editing activity. We hypothesized that *Mycoplasma* relies on this pretransfer editing activity to maintain a threshold level of fidelity that is required by this pathogen, albeit it is not sufficient to fully suppress mischarging and protect the proteome from statistical substitutions.

X-Ray Crystal Structure of *M. mobile* LeuRS-LeuAMS Is Similar to Other LeuRSs. *M. mobile* LeuRS was cocrystallized as a dimer in two conformations with a leucyl-sulfamoyl-adenylate analog (Leu-AMS) of leucyl adenylate in the space group R32 and diffracted to 2.07 Å. One of the conformers (Fig. 3*A*) had weak density representing the mobile C-terminal domain, which was absent in the other conformer. Although tRNA was present in the crystallization medium, neither of the LeuRS conformers contained bound tRNA.

The overall structure of the *M. mobile* LeuRS enzyme is similar to structures determined for *Thermus thermophilus* LeuRS (27) (TtLeuRS, PDB ID code 1H3N) and *E. coli* LeuRS (29) (EcLeuRS, PDB ID code 4AQ7), despite that the native *M. mobile* LeuRS is missing CP1 and leucine-specific domains (6) (Fig. S3). This supports the modular nature of aaRS expansion and provides a glimpse of the aminoacylation core in its ancient state. In *T. thermophilus* and *E. coli* LeuRSs, two β-strands link the core of the enzyme to the CP1 domain that is responsible for

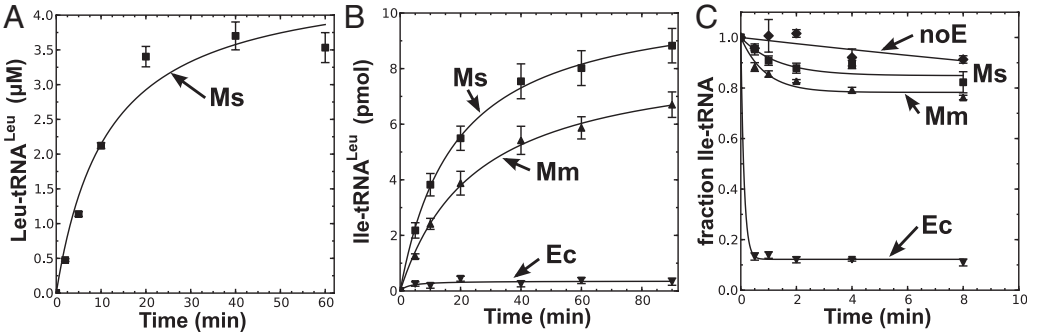

Fig. 1. *M. synoviae* LeuRS mischarges tRNA^{Leu}. (*A*) Leucylation by *M. synoviae* LeuRS was carried out by using 21 μM [^{14}C]leucine (50 μCi/mL), 4 μM tRNA^{Leu}, and 100 nM enzyme. (*B*) Isoleucine mischarging by *M. synoviae* LeuRS incorporated 40 μM [^{3}H]isoleucine (166 μCi/mL), 4 μM tRNA^{Leu}, and 1 μM enzyme. *M. mobile* LeuRS was used as a positive control. (*C*) Deacylation reactions for *M. synoviae* LeuRS contained ~6.5 μM [^{3}H]Ile-tRNA^{Leu} and 100 nM enzyme. *E. coli* LeuRS was used as the positive control. Abbreviations are as follows: ■, *M. synoviae* LeuRS (Ms); ▲, *M. mobile* LeuRS (Mm); ▼, *E. coli* LeuRS (Ec); and ◆, no enzyme control (noE). Error bars represented SDs calculated from triplicated reactions.

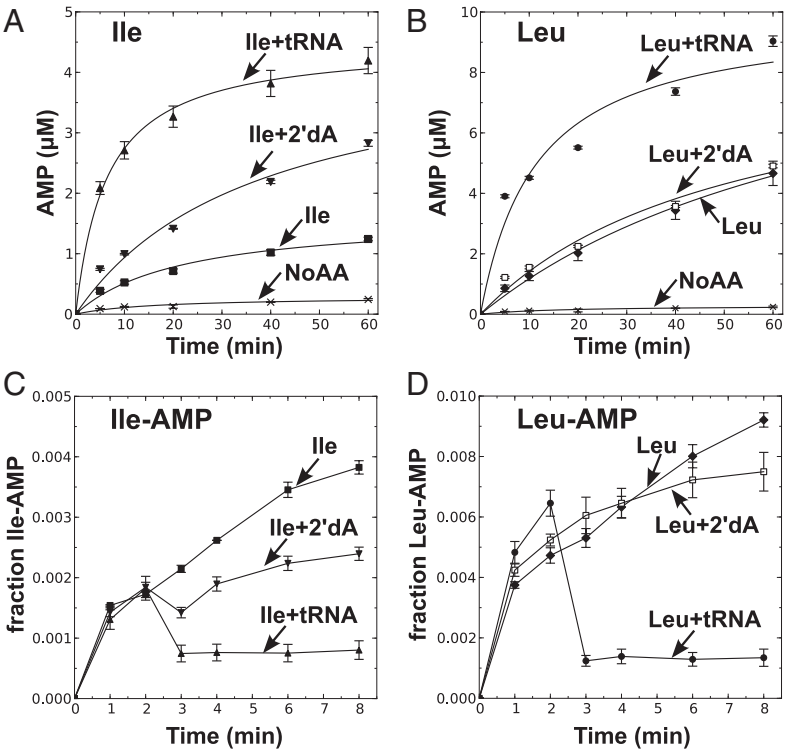

Fig. 2. *M. mobile* LeuRS exhibits pretransfer editing activity. Reaction mixtures contained 10 μM *M. mobile* $tRNA^{Leu}$ or a tRNA analog with 2′-deoxyadenosine substituted for the A_{76} nucleotide (2′dA-tRNA) and 1 μM *M. mobile* LeuRS. Amino acid-dependent AMP formation is measured by TLC of reaction aliquots from ATPase reactions with 5 mM isoleucine (*A*) or 5 mM leucine (*B*). Aminoacyl-adenylate was measured with 5 mM isoleucine (*C*) or 5 mM leucine (*D*). Fractions indicated by the *y* axis represent the intensity of the spot representing [^{32}P]adenylate divided by the total intensity of ^{32}P in the lane. Abbreviations are as follows: ■, isoleucine only (Ile); ▲, isoleucine and tRNA (Ile+tRNA); ▼, isoleucine and 2′dA-tRNA (Ile+2′dA); ◆, leucine only (Leu); ●, leucine and tRNA (Leu+tRNA); □, leucine and 2′dA-tRNA (Leu+2′dA); and ×, no amino acid control (No AA). Error bars represent SDs derived from triplicated reactions.

posttransfer editing. Significantly, although *M. mobile* LeuRS is missing its CP1 domain, the two β-strands (residue Gly226 to Lys237), now connected by a dipeptide (Asp-Gly), are retained and structurally conserved. These overlap with the N-terminal β-strand Arg226 to Gly229 and C-terminal β-strand Lys412 to Tyr415 from *E. coli* LeuRS. The structure model for the C-terminal domain is based on weak, but unambiguous, electron density present in difference Fourier maps. The C-terminal domain is linked to the core of the enzyme by a flexible tether (11) and interacts with the corner of the L-shaped tRNA. Thus, stabilization of the position of this mobile domain in either the editing or aminoacylation complex requires tRNA binding (29).

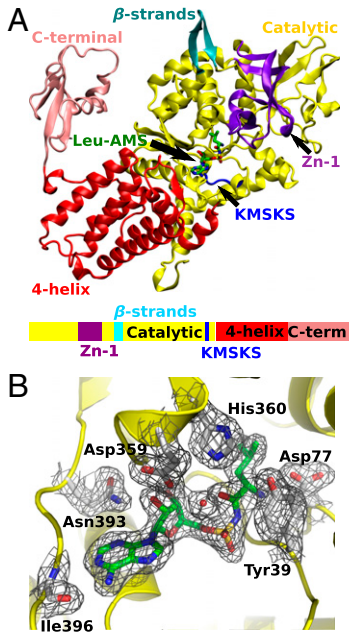

Fig. 3. X-ray crystal structure of *M. mobile* LeuRS Leu-AMS complex. (*A*) A ribbon diagram of the protomer with electron density of C-terminal domain is colored as follows: catalytic domain, yellow; four-helix bundle domain, red; C-terminal domain, pink; Zn binding domain, purple; KMSKS loop, blue; two linking β-strands, cyan. The Leu-AMP analog, Leu-AMS, is shown in stick model. A color-coordinated cartoon of the primary sequence is shown below the structure. (*B*) The $2F_o$-F_c electron density map of the aminoacylation active site is contoured at 1.0 σ (black mesh). The Leu-AMS is highlighted in green, and interacting amino acid residues are labeled and shown in gray.

The aminoacylation core of the protein is intact, and the adenylate analog Leu-AMS is bound to the enzyme active site (Fig. 3*B*). *M. mobile* LeuRS and *T. thermophilus* LeuRS (27) rely upon similar sets of amino acid residues to bind Leu-AMS in their active sites (Fig. S3*A*), suggesting that the adenylate is stabilized by conserved interactions within the canonical LeuRS core. The flexible KMSKS loop responsible for ATP binding partially covers the active site entrance in a manner similar to the semiopen conformation of this signature sequence in *E. coli* and *T. thermophilus* LeuRSs (27, 29). Compared with the *E. coli* LeuRS aminoacylation complex (Fig. S3*C*), a conserved tyrosine residue undergoes significant conformational change upon tRNA binding. The phenyl ring of Tyr39 in *M. mobile* LeuRS adopts a conformation similar to that of Tyr43 in *T. thermophilus* LeuRS (27) (Fig. S3*B*) to protect the adenylate from nonproductive hydrolysis. In contrast, in the *E. coli* LeuRS aminoacylation complex, the side chain of the homologous tyrosine is flipped outward by about 120° to accommodate binding of the terminal tRNA A_{76} (29) (Fig. S3*D*).

CP1 Insertions Stimulate Pretransfer Editing in *M. mobile* LeuRS. It has been proposed that a primitive translation machinery lacked modern specificity and fidelity mechanisms, resulting in statistical protein synthesis (33). As fidelity requirements increased during evolution, the canonical aminoacylation core was fused to a CP1 domain to accommodate more stringent amino acid specificity requirements. Despite its unusual lack of a CP1 domain, the solved crystal structure of *M. mobile* LeuRS supports that it has retained a canonical aminoacylation core as well as faithful interactions with the adenylate intermediate that mimics a typical

LeuRS. Thus, we hypothesized that we can use *M. mobile* LeuRS as a scaffold to reconstruct evolutionary events that adapted the aaRS for increased accuracy. As such, the CP1 domain from *E. coli* LeuRS was fused to *M. mobile* LeuRS (MmLeuRS/CP1^{Leu}) (30). We also constructed hybrids using the CP1 domains from IleRS (MmLeuRS/CP1^{Ile}) and ValRS (MmLeuRS/CP1^{Val}) (30). Each of the hybrid LeuRSs displayed leucylation activities comparable to the wild-type *M. mobile* enzyme (30) (Fig. 4*A*), supporting that the LeuRS core domain in these hybrid proteins retained its structural integrity. Significantly and regardless of their specificities, these inserted CP1 domains suppressed LeuRS mischarging of isoleucine (Fig. 4*B*), methionine (Fig. 4*C*), and valine (Fig. 4*D*).

We asked whether the addition of these CP1 domains stimulated tRNA-dependent pretransfer editing of the *M. mobile* LeuRS active site. We performed amino acid-dependent ATP hydrolysis assays with *M. mobile* ddA-tRNA^{Leu}. Compared with wild-type *M. mobile* LeuRS (Fig. 5*A*), all three CP1 fusion enzymes displayed increased tRNA-dependent AMP production in the presence of isoleucine (Fig. 5 *B–D*). The cognate leucine substrate failed to increase tRNA-dependent AMP formation activity (Fig. S4 *A–D*). Significantly, although the amplitude of the stimulation of pretransfer editing varied depending on the hybrid, it occurred regardless of the origin and specificities of the fused CP1 domains.

Degenerate *M. synoviae* CP1 Domain Confers Fidelity to *M. mobile* LeuRS via Enhanced Pretransfer Editing Activity. Because *M. synoviae* LeuRS produces mischarged tRNAs (Fig. 1*B*) similar to *M. mobile* LeuRS, we asked whether insertion of an editing-defective degenerate CP1 domain would also influence pretransfer editing activity of *M. mobile* LeuRS. We generated a hybrid *M. mobile* LeuRS that contained the degenerated CP1 domain from *M. synoviae* LeuRS (MmLeuRS/Ms^{Leu}). This fusion enzyme robustly aminoacylated in vitro transcribed *M. mobile* tRNA^{Leu} (Fig. 6*A*). Surprisingly, the editing-defective *M. synoviae* CP1 domain also suppressed mischarging (Fig. 6*A*).

We asked whether this increased fidelity could be attributed to increases in amino acid discrimination in the aminoacylation site (30) or enhanced pretransfer editing activity, or a combination of both mechanisms. Whereas the k_{cat} was similar for leucine ($11 \pm 2\ s^{-1}$) and isoleucine ($9 \pm 2\ s^{-1}$), PP_i exchange assays showed that the K_m was, respectively, 0.03 ± 0.01 mM and 2.7 ± 0.7 mM, resulting in a discrimination factor of just 1/111. Thus, unlike fusion of the *E. coli* LeuRS and IleRS CP1 domains that enhanced amino acid discrimination of the *M. mobile* LeuRS (30), the MmLeuRS/Ms^{Leu} mimics fusion of the ValRS CP1 domain. As such, the chimeric *Mycoplasma* LeuRS exhibited an enhanced pretransfer editing activity (Fig. 6*B*) that cleared misactivated isoleucine, but not cognate leucine (Fig. S4*E*). Because pretransfer editing has been associated with the canonical core of LeuRS (21), we hypothesize that the linkage or connectivity between the aminoacylation active site and CP1 domain is more important to adenylate clearance as opposed to the nature of the independent CP1 domain.

Discussion

Establishing and maintaining the genetic code requires faithful translation of the codon message to its cognate amino acid (34). In modern cells, this is facilitated by exquisite quality-control mechanisms that ensure error correction when necessary for misaminoacylation. Primitive translation machineries that coevolved in a chaotic environment with an emerging genetic code were likely error-prone and produced statistical proteins (33). Indeed, urzymes, a rationally designed minimal construct of a class I aaRS active site that retains catalytic activity, exhibit decreased specificity for cognate amino acids (35, 36). This supports that evolutionary addition of auxiliary domains enhanced substrate specificity.

Sequence- and structure-based phylogenetic analyses demonstrated that aaRSs originate from their canonical aminoacylation core (37, 38). Thus, these early translation mechanisms would have been dependent on aaRS progenitors composed simply of their core domains to aminoacylate tRNA. In the case of the class I aaRSs, the core is a Rossmann ATP binding fold that is distributed widely throughout many protein families. The class II aaRS core fold is much more rare (39–41). As the cell and its genetic code became more sophisticated, domains responsible for clearing errors were added in both aaRS classes to provide the necessary quality control for translation.

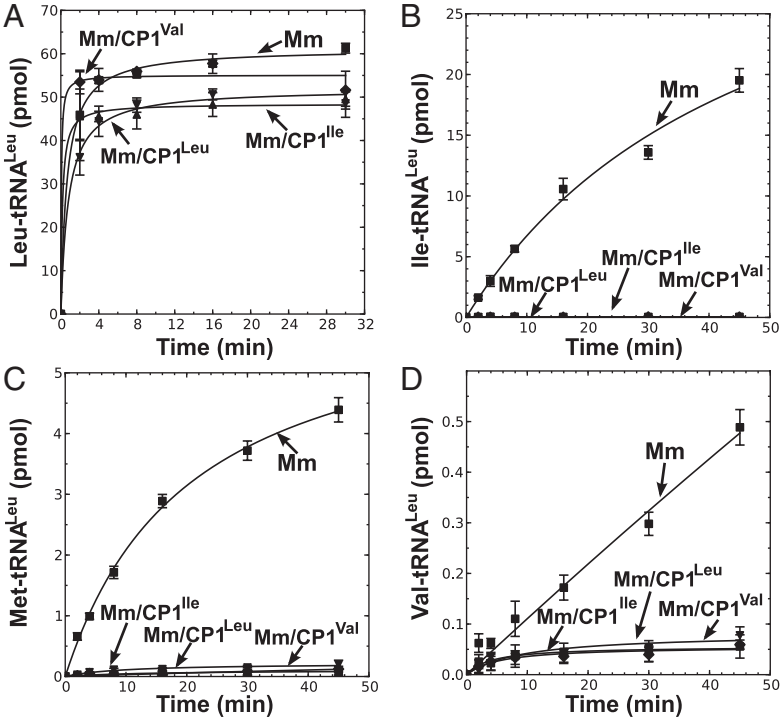

Fig. 4. Aminoacylation and misaminoacylation activities of *M. mobile* LeuRSs that contain hybrid CP1 domains. Aminoacylation of 4 μM tRNA^{Leu} in presence of (*A*) 21 μM [^{3}H]leucine (318 μCi/mL), (*B*) 21 μM [^{3}H]isoleucine (166 μCi/mL), (*C*) 20.2 μM [^{35}S]methionine (115 μCi/mL), and (*D*) 20 μM [^{14}C]valine (259 μCi/mL). Symbols used are as follows: ■, MmLeuRS (Mm); ▲, MmLeuRS/CP1^{Leu} (Mm/CP1^{Leu}); ▼, MmLeuRS/CP1^{Ile} (Mm/CP1^{Ile}); and ◆, MmLeuRS/CP1^{Val} (Mm/CP1^{Val}). Error bars represent SDs from triplicated reactions.

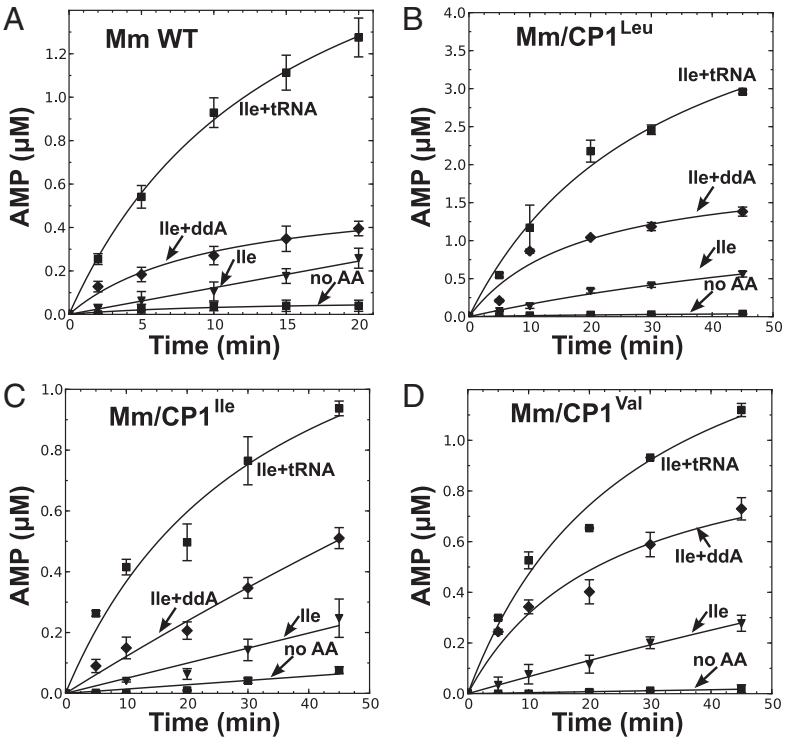

Fig. 5. CP1 addition to hybrid *M. mobile* LeuRS enhances pretransfer editing. Reaction mixture contained 1 μM enzyme, 10 μM $tRNA^{Leu}$, or $tRNA^{Leu}$ with A_{76} replaced by dideoxyadenosine (ddA), 18.1 μM [α-^{32}P]ATP (40 μCi/mL), and 2.5 mM isoleucine. The AMP formation activities are measured for Mm LeuRS (*A*), MmLeuRS/$CP1^{Leu}$ (Mm/ $CP1^{Leu}$) (*B*), MmLeuRS/$CP1^{Ile}$ (Mm/$CP1^{Ile}$) (*C*), and MmLeuRS/$CP1^{Val}$ (Mm/ $CP1^{Val}$) (*D*). Symbols used are as follows: ◆, reaction without amino acid present (no AA); ▲, isoleucine (Ile); ▼, isoleucine with tRNA (Ile+tRNA); and ■, isoleucine with ddtRNA (Ile+ddA). Error bars represent the SD values based on three separate experiments.

LeuRS, IleRS, and ValRS rely on a conserved CP1 tRNA deacylase domain to clear mischarged tRNA (9, 13) before they are released to elongation factor-Tu for delivery to the ribosome. Previously, we showed that we can remove the CP1 domain from yeast mitochondrial and *E. coli* LeuRS and maintain complete fidelity (21). In these two LeuRS cases, the CP1 domain acted as a rheostat to dampen pretransfer editing. Deletion of the CP1 domain unmasked a tRNA-dependent pretransfer editing mechanism for LeuRS that was associated with the canonical core of the enzyme. Pretransfer editing clears the misactivated adenylate before the noncognate amino acid can be transferred to the tRNA. Because of its association with the ancient aminoacylation core, we hypothesize, then, that pretransfer editing might have served as an ancient quality-control mechanism.

Herein, we demonstrated that two *Mycoplasma* LeuRSs also harbor pretransfer editing activity. In these two natural examples, however, the pretransfer editing activity is insufficient to protect mischarging and results in errors that are translated during protein synthesis. Remarkably, addition of the CP1 domain—regardless of its origin from a LeuRS, IleRS, or ValRS—again served as a molecular rheostat, but to increase pretransfer editing activity.

It is clear that *Mycoplasma* capitalized on the rheostat capabilities of the LeuRS CP1 domain to influence translational fidelity. It is possible that producing statistical proteins is strategically embraced by these *Mycoplasma* pathogens to evade host immune systems (6). This inherent pretransfer editing activity in the canonical core also supports that a primitive LeuRS (before CP1 domain insertion) likely contained this as a rudimentary fidelity mechanism that was sufficient to provide a threshold level of fidelity for an early cell as well as enough charged $tRNA^{Leu}$ to allow an ancient organism to prosper.

The homologous CP1 domains have adapted for different specificities to clear noncognate amino acid, which idiosyncratically challenge the fidelity of LeuRS, IleRS, and ValRS. In LeuRS and ValRS, the CP1 domains have evolved to efficiently execute posttransfer editing. The balance of fidelity mechanisms that rely on post- or pretransfer editing can differ and shift (8, 25). A preponderance of posttransfer editing versus pretransfer editing can be dictated by a rapid aminoacyl-transfer step in the aminoacylation site (22, 23, 41). Likewise, the CP1 domain in IleRS relies upon pretransfer editing as its dominant editing pathway (42) because of a slow transfer rate (22).

Our recent work revealed that fusion of the CP1 domains to *M. mobile* LeuRS can improve amino acid selectivity for aminoacylation (30). Here, we showed that fusion of CP1 domains from different specificities and origins can stimulate pretransfer editing activity in *M. mobile* LeuRS, albeit at different levels. Regardless, this enhanced pretransfer editing suppresses mischarging independently (MmLeuRS/$CP1^{Val}$ and MmLeuRS/Ms^{Leu}) or synergistically with an enhancement of selectivity in the aminoacylation site (MmLeuRS/$CP1^{Leu}$ and MmLeuRS/$CP1^{Ile}$) (30). It is possible that the CP1 insertion favored an active site

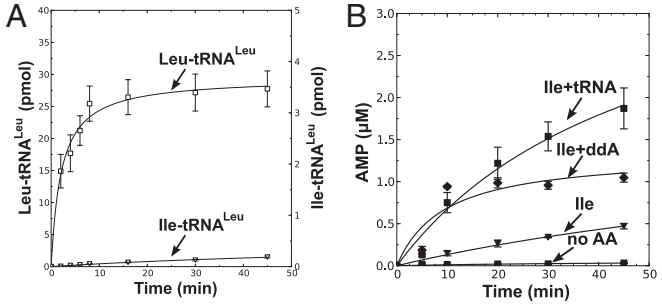

Fig. 6. *M. synoviae* LeuRS CP1 addition to *M. mobile* LeuRS suppresses mischarging via enhanced pretransfer editing activity. (*A*) Aminoacylation and misaminoacylation activity of Mm/Ms LeuRS was carried out with 4 μM $tRNA^{Leu}$ in the presence of 21 μM [^{3}H]leucine (318 μCi/mL) with 1 μM enzyme or 21 μM [^{3}H]isoleucine (97 μCi/mL) with 1 μM enzyme. Symbols used are as follows: □, Leu-$tRNA^{Leu}$ and △, Ile-$tRNA^{Leu}$. (*B*) The AMP formation activity was measured for Mm/Ms LeuRS using 1 μM enzyme, 10 μM $tRNA^{Leu}$, or $tRNA^{Leu}$ with A_{76} replaced by dideoxyadenosine (ddA-tRNA), 18.1 μM [α-^{32}P] ATP, and 2.5 mM isoleucine. Symbols used are as follows: ◆, reaction without amino acid present (no AA); ▲, isoleucine (Ile); ▼, isoleucine with tRNA (Ile+tRNA); and ■, isoleucine with ddA-tRNA (Ile+ddA). Error bars represent the SD values based on three separate experiments.

conformation more suitable for pretransfer editing and amino acid selection. In many cases, this activity seems to be enhanced in the presence of tRNA (20, 21, 23). Thus, in the evolution of LeuRS, IleRS, and ValRS, the original insertion of the CP1 domain likely affected fidelity via a set of mechanisms. Indeed, we hypothesize that the CP1 domain addition used a rheostat-like function as a selective advantage to potentiate amino acid selection and pretransfer editing activity in the aminoacylation core, while introducing a posttransfer editing hydrolytic active site.

Materials and Methods

Crystallization and X-Ray Diffraction. *M. mobile* LeuRS was crystallized in 0.1 M *bis*-Tris (pH 5.5), 0.6 M ammonium acetate, and 20% (vol/vol) PEG3350 at 4 °C, via hanging drop vapor diffusion. Drops combined 1 μL of mother liquor and 1 μL of protein solution at 10 mg/mL LeuRS mixed with 2 mM LeuAMS and 40 μM *M. mobile* $tRNA^{Leu}$. Crystals diffracting to 2.07 Å were grown for 3 mo. The crystals were soaked for 10 s in mother liquor supplemented with 15% (vol/vol) glycerol, mounted on CrystalCap HT cryoloops (Hampton Research), and vitrified in liquid nitrogen.

Diffraction data were collected at an insertion device beam line (21-ID-F) using Mar 225 CCD detector (LS-CAT sector; Advanced Photon Source). Data were integrated and scaled using the HKL-2000 (43) package. Molecular replacement was carried out using *E. coli* LeuRS (PDB ID code 4AQ7) as a model with Phaser (44). Manual model rebuilding was carried out as described in *SI Materials and Methods*. Data collection and refinement statistics are provided in Table S1.

Enzyme Assays. ATPase assays containing 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 10 μM *M. mobile* $tRNA^{Leu}$ or unchargeable analog, 18 μM radiolabeled ATP, and 1 μM enzyme were initiated with 2.5 mM amino acid. Reaction aliquots (2 μL) were quenched by spotting on PEI-cellulose TLC plates. The plates were developed in 750 mM KH_2PO_4 (pH 3.5) and analyzed by phosphorimaging.

We adapted a method (45) to isolate adenylate. To accumulate adenylate, the ATPase assay reaction was allowed to react for 2 min, after which 10 μM of *M. mobile* $tRNA^{Leu}$ or its unchargeable analog was added to the reaction buffer. Aliquots of 1.5 μL were quenched in 3 μL of 0.1% SDS and 50 mM sodium acetate (pH 5.0) and spotted on PEI-cellulose TLC plates, which were developed in 0.1 M ammonium acetate and 5% acetic acid at 25 °C and analyzed by phosphorimaging.

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