Structural dynamics of the aminoacylation and proofreading functional cycle of bacterial leucyl-tRNA synthetase

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Leucyl-tRNA synthetase (LeuRS) produces error-free leucyl-tRNA\textsuperscript{Leu} by coordinating translocation of the 3′ end of (mis-)charged tRNAs from its synthetic site to a separate proofreading site for editing. Here we report cocrystal structures of the \textit{Escherichia coli} LeuRS–tRNA\textsuperscript{Leu} complex in the aminoacylation or editing conformations, showing that translocation involves correlated rotations of four flexibly linked LeuRS domains. This pivots the tRNA to guide its charged 3′ end from the closed aminoacylation state to the editing site. The editing domain unexpectedly stabilizes the tRNA during aminoacylation, and a large rotation of the leucine-specific domain positions the conserved KMSKS loop to bind the 3′ end of the tRNA, promoting catalysis. Our results give new insight into the structural dynamics of a molecular machine that is essential for accurate protein synthesis.

LeuRS is a large, multidomain, class 1a aminoacyl-tRNA synthetase (aaRS) whose essential function in all organisms is to synthetize Leu-tRNA\textsuperscript{Leu} for use in protein synthesis. Similar to several other synthetases, notably the other two class 1a synthetases, valyl-tRNA synthetase (ValRS) and isoleucyl-tRNA synthetase (IleRS), LeuRS possesses an error-correction mechanism to enhance the specificity of aminoacylation and, thus, the accuracy of protein synthesis. This post-transfer editing mechanism hydrolytically deacylates tRNA\textsuperscript{Leu} that has been mischarged with noncognate amino acids that are similar to leucine, such as isoleucine, methionine or noncanonical norvaline\textsuperscript{1}.

LeuRS comprises a main enzyme body (Rossmann-fold catalytic domain and class 1a anticodon-binding domain) and four flexibly linked additional domains, denoted the zinc (ZN1), editing, leucine-specific and C-terminal domains (Fig. 1a). Proofreading requires that the 3′ end of the tRNA, which is initially charged (or mischarged) in the so-called ‘synthetic’ site of the enzyme, translocate to the editing site, located ~35 Å away in an independently folded editing, or CP1, domain\textsuperscript{2}. Previously, we and others have characterized various structural and biochemical features of the editing state of LeuRS. These include (i) determination of the crystal structure of the \textit{Thermus thermophilus} LeuRS (LeuRSTT) tRNA\textsuperscript{Leu} in complex with the 3′ end of the tRNA bound in the editing site\textsuperscript{3}, (ii) elucidation of the structural basis for the amino acid specificity of the LeuRS editing site, which binds noncognate amino acids but rejects cognate leucine\textsuperscript{2} and (iii) a theoretical analysis of the hydrolytic mechanism\textsuperscript{4}. Recently, a series of benzoxaborole compounds were shown to bind specifically in the LeuRS editing site in a tRNA-dependent fashion\textsuperscript{5}. These compounds form a long-lived covalent adduct with the tRNA 3′-terminal ribose hydroxyl groups in the editing site, thus trapping the tRNA on the enzyme and inhibiting aminoacylation\textsuperscript{5}. Benzoxaboroles have the potential to be potent antibiotics and are currently undergoing development for use against fungal, bacterial and parasitic pathogens\textsuperscript{5–7}.

Although the editing states of bacterial class 1a LeuRS\textsuperscript{5}, ValRS\textsuperscript{9} and IleRS\textsuperscript{10} are well characterized, the only published structure of any class 1a synthetase with tRNA bound in the aminoacylation active site is that of an archaeal LeuRS from \textit{Pyrococcus horikoshii} (LeuRSPH)\textsuperscript{11}. However, archaeal and eukaryotic cytoplasmic LeuRSs are architecturally distinct from bacterial LeuRS\textsuperscript{12,13}. Therefore, the available structures of the editing and aminoacylation states, being, respectively, from bacterial and archaeal systems, are not directly comparable. Furthermore, the LeuRSPH–tRNA\textsuperscript{Leu} aminoacylation complex lacks any bound small substrates and does not represent the enzymatically active aminoacylation state. Indeed, few class 1 synthetase–tRNA complexes have been determined in this functional state, the only examples being the class 1b GlnRS\textsuperscript{14–16} and GluRS\textsuperscript{17,18} aminoacylation complexes. In other class I cocrystal structures, either the tRNA is directed to the editing site\textsuperscript{8,10}, the 3′ end is disordered or incorrectly bound\textsuperscript{19–24}, or the adenylate is not present\textsuperscript{25}.

Here we present the crystal structure of the functional aminoacylation complex of \textit{E. coli} LeuRS (LeuRSEC). In this ternary complex structure, the 3′ end of \textit{E. coli} tRNA\textsuperscript{Leu}(UAA) is bound in the synthetic site and poised to interact with leucyl-adenylate (present as a nonhydrolyzable analog), the enzyme-bound, activated intermediate of the two-step aminoacylation reaction (Fig. 1b). In addition,
we present the high-resolution structure of LeuRSEC with the tRNA 3′ end bound in the editing site (Fig. 1c) and leucine or leucyl-adenylate analog bound in the synthetic site. We also report high-resolution structures of two different crystal forms of the complex with the tRNA trapped in the editing site by the simplest benzoxaborole compound 1-hydroxy-3H-2,1-benzoxaborole. Comparison of these structures enables us to describe the substantial domain and active site rearrangements that accompany tRNA translocation between the two functional states, providing new insight into the mechanism of aminoacylation and proofreading in class 1a synthetases.

RESULTS
Summary of structures determined
We subjected complexes of LeuRSEC with a tRNA5Leu(UAA) transcript (Supplementary Fig. 1a, b) and various combinations of small-molecule substrates and inhibitors, such as leucine, leucinol, ATP, AMP, LeuAMS (the sulfamoyl analog of leucyl-adenylate, LeuAMP) and 1-hydroxy-3H-2,1-benzoxaborole, to extensive crystallization screens with the aim of obtaining crystals of different functional states of the enzyme. We obtained two different crystal forms, both diffracting to up to 2-Å resolution, with the tRNA directed toward or bound in the editing site. These also grew with or without 1-hydroxy-3H-2,1-benzoxaborole bound in the editing site (where it makes a covalent adduct with the 3′ end of the tRNA5) and with or without leucine or LeuAMS bound.

Table 1 Data collection and refinement statistics

|                  | Editing + leucine | Editing + benzoxaborole | Editing + benzoxaborole | Aminoacylation + LeuAMS |
|------------------|-------------------|-------------------------|-------------------------|-------------------------|
| **Data collection** |                   |                         |                         |                         |
| Space group      | P2_12_12_1        | P2_12_12_1              | P2_1                    | C2                      |
| Cell dimensions  |                   |                         |                         |                         |
| a, b, c (Å)      | 77.08, 119.37, 141.10 | 76.18, 118.94, 141.03 | 89.68, 77.11, 91.14    | 158.66, 69.20, 228.84  |
| α, β, γ (°)      | 90.00, 90.00, 90.00 | 90.00, 90.00, 90.00     | 90.00, 102.24, 90.00   | 90.00, 104.35, 90.00   |
| Resolution (Å)² | 50–2.00 (2.00–2.10) | 50–2.08 (2.08–2.15)     | 50–2.02 (2.02–2.09)    | 50–2.5 (2.50–2.60)     |
| Rsym             | 7.1 (71.6)        | 5.6 (36.7)              | 6.6 (68.8)              | 8.4 (51.5)              |
| l / al           | 10.5 (2.0)        | 20.1 (3.1)              | 14.2 (1.6)              | 11.1 (2.1)              |
| Completeness (%) | 99.3 (99.1)       | 96.7 (74.2)             | 98.2 (84.5)             | 90.3 (76.6)             |
| Redundancy       | 3.90 (3.76)       | 3.91 (2.80)             | 3.65 (2.81)             | 2.89 (2.16)             |
| **Refinement**   |                   |                         |                         |                         |
| Resolution (Å)   | 2.0               | 2.08                    | 2.02                    | 2.5                     |
| No. reflections (work/free) | 83,506 / 4,411 | 71,134 / 3,759         | 74,330 / 3,977         | 71,885 / 3,826         |
| Rwork / Rfree   | 0.210 / 0.257     | 0.199 / 0.244           | 0.208 / 0.249           | 0.188 / 0.250           |
| No. atoms        |                   |                         |                         |                         |
| Protein          | 6,465             | 6,517                   | 6,476                   | 2 × 6,836               |
| tRNA             | 1516              | 1711                    | 1777                    | 2 × 1,653               |
| Ligand           | 9 (Leucine)       | 9 (AN2679)              | 9 (AN2679)              | 64 (2 × LeuAMS)         |
| Mg²⁺             | 1                 | 2                       | 1                       | 2                       |
| Water/other      | 415               | 325/4 glycerol          | 402                     | 169                     |
| **R.m.s. deviations** |                 |                         |                         |                         |
| Bond lengths (Å) | 0.014             | 0.011                   | 0.013                   | 0.014                   |
| Bond angles (°)  | 1.68              | 1.50                    | 1.64                    | 1.77                    |

*Values in parentheses are for highest-resolution shell. \( ^{1} \)Values are for each molecule in the asymmetric unit, with chain indicator given in square brackets.
in the synthetic site. A different condition gave crystals diffracting to 2.5-Å resolution of the LeuRS–tRNA\textsubscript{Leu}(UAA)–LeuAMS ternary complex with the tRNA in the aminoacylation conformation (Supplementary Fig. 1c). Details of the structure determination are given in the Online Methods and Table 1.

**The LeuRS–tRNA\textsubscript{Leu}–LeuAMS aminoacylation complex structure**

The *E. coli* LeuRS–tRNA\textsubscript{Leu}–LeuAMS ternary complex (Fig. 1b) shows the 3′ end of the tRNA bending back into the synthetic active site, bringing the 2′-OH of the A76 ribose into the required position to attack the nonreactive leucyl-adenylate analog; thus, the structure closely mimics the functional aminoacylation state. Notably, in only this state are all four flexibly linked domains of the enzyme fully ordered because of a complex network of mutually stabilizing interdomain and protein–tRNA interactions. Owing to extra contacts between bases 69–76 of the tRNA 3′ strand to multiple domains of LeuRSEC, the tRNA makes considerably more contacts with the synthetase in the aminoacylation state than in the editing state (Fig. 2a, Supplementary Fig. 1a,b and Supplementary Table 1). These extra contacts not only ensure correct selection of cognate tRNAs but also control the domain rearrangements that create the catalytically active aminoacylation state. In the editing state, discrimination of cognate tRNA is no longer essential, and the key A73 identity element\textsuperscript{26,27} is not recognized by the protein.

The hairpin structure of the tRNA 3′ end is stabilized by both protein–tRNA and intra-tRNA interactions, the latter including base-base stacking and phosphate-base hydrogen bonds. A rotamer flip of the class 1a conserved tyrosine\textsuperscript{28}, Tyr43, into an open position avoids a steric clash with the bound ribose of A76. This tyrosine acts as a lid that can either pack down on the hydrophobic amino acid substrate, preventing premature hydrolysis of the leucyl-adenylate, aminoacylation but not the editing state (see below), has a mutually stabilizing interface with the first four residues of the editing domain loop 286–298, also disordered in the editing state. Unexpectedly, a short helix in this loop stacks upon the G1-U72 base pair of the tRNA, which remains unbroken (Fig. 2b). Residue Glu292 in this helix forms a hydrogen bond with the phosphate of A73 and the 2′-OH of U72, and also forms a salt bridge with Arg416, which is part of the motif mentioned earlier. These interactions, respectively, block the acceptor stem in place and form a physical barrier separating the single-stranded region 73–76 from the acceptor-stem helix (Fig. 2a).

Indeed, for the 3′ end of the tRNA to translocate from the synthetic site into the editing site, the Gla292-Arg416 salt bridge would need to be broken. The unexpected observation that the CP1 editing domain loop 286–298 is directly involved in positioning the tRNA 3′ end of tRNALeu (see also Supplementary Fig. 4a,b), (d) interactions of the C-terminal domain with the T-loop, D-loop and long variable arm of the tRNA (surface representation) are conserved in the editing and aminoacylation states (overlaid).

or be open, to allow A76 binding and the aminoacylation reaction to proceed.

The discriminator base A73 is stacked between Trp223 and Arg416, making base-specific hydrogen bonds to the main chain of Arg416 and Arg418, which are part of the highly conserved (discussed later) 416-RLRWQGVSQRYWQ-429 motif that links the editing domain to the catalytic domain\textsuperscript{3,11}. Other residues from this motif also interact with the tRNA, whereas C74 is flipped out into a pocket formed between the ZN1 domain and the catalytic domain (Fig. 2a). The ZN1 domain, ordered in the aminoacylation complex. (**a**) Several domains (color coded as in Fig. 1) of LeuRS are involved in binding and stabilizing the conformation of nucleotides 69–76 of the 3′ end of tRNA\textsubscript{Leu} (green). The base of G71 is omitted for clarity. (**b**) The α-helix at residues 291–298 of the editing domain stacks on the G1-U72 base pair and contacts the backbone of A73. (**c**) A network of interactions from the anticodon-binding domain, conserved between the two states (overlaid), specifically recognizes the base of U16 (see also Supplementary Fig. 4a,b). (**d**) Interactions of the C-terminal domain with the tRNA make considerably more contacts with the syn-
the 416-R/KLRDWGVSQRWYG-429 motif is highly conserved (Supplementary Fig. 2c), as are ZN1 domain residues Asn168, Glu169 and Gln190, which interact with the tRNA (not shown). The alignment also suggests that the editing-domain loop containing the helix that stacks on the first base pair of the tRNA is structurally, and probably functionally, conserved, although the sequence can diverge (Supplementary Fig. 2b). A glutamate or aspartate residue is found at position 292 in about half the sequences, suggesting that the 292–416 salt bridge is not essential.

Structure of the E. coli LeuRS–tRNA^Leu editing complex
The overall structure of the E. coli LeuRS–tRNA^Leu editing complex, determined at nearly 2-Å resolution in the orthorhombic form, is shown in Figure 1c. The orthorhombic and monoclinic forms of the complex have only minor differences in domain orientations, and the occurrence of similar structures in two different crystal forms suggests that they faithfully represent the post-transfer editing state of the enzyme. This is reinforced by the global similarity (apart from slight changes in the orientation of the flexibly linked editing and C-terminal domains) of the LeuRSEC editing complex to the previously published structure of the LeuRSTT editing complex (PDB 2BYT) (Supplementary Fig. 3). However, there are some notable differences between the two editing complexes. First, the ZN1 domain is well ordered and closes over the leucine binding site in the LeuRSTT complex, whereas there is only weak, interpretable electron density for the corresponding region in the LeuRSEC editing complex. This flexibly linked domain has a more active role in the LeuRSEC aminoacylation complex, as described earlier. Second, the LeuRSEC leucine-specific domain is larger and has a different topology compared to that of LeuRSTT. Only in the LeuRSEC editing complex does the leucine-specific domain directly interact with the tRNA. This occurs via an extended β-hairpin, absent from LeuRSTT, that contacts bases 10 and 27 via Arg595 and Arg600 (Supplementary Fig. 3). Owing to the high variability in the sequence of the leucine-specific domain (and its absence in some cases), these contacts are likely to be idiosyncratic for E. coli and other closely related bacterial LeuRS enzymes.

A second notable difference in protein–tRNA interactions concerns tRNA base 16, which is not contacted in the T. thermophilus system. However, in both the aminoacylation and editing complexes of LeuRSEC, U16 is involved in a network of direct and water-mediated interactions with Lys711 (K), Asp714–Asp715 (DD) and Arg718–Arg719 (RR), referred to as the K/DD/RR motif (Fig. 2c and Supplementary Fig. 4a). Mutations of these residues reduce tRNA binding and catalytic efficiency for aminoacylation, and substitution of U16 by guanine or cytosine eliminates aminoacylation (Supplementary Fig. 4b,c). This suggests that U16 is a previously overlooked identity element, at least for E. coli LeuRS and related bacteria in which the K/DD/RR motif is conserved (Supplementary Fig. 2d). Phylogenetic analysis shows that U16 is conserved not only in all E. coli tRNA^Leu isoacceptors but also in those of most bacteria. Uridine is more favored than cytosine, which occurs occasionally, by the interaction of its O4 with well-conserved Lys711 (Supplementary Fig. 4a).

In the LeuRSTT-tRNA editing complex, we used a tRNA^Leu with a truncated long variable arm comprising two base pairs and a tetraloop (Supplementary Video 1). In both LeuRSEC conformations, the long variable arm is as in the wild type (four base pairs and a tetraloop) and contacts the C-terminal domain via the variable-stem bases P47f-P47i (Fig. 2d and Supplementary Fig. 1). In all LeuRSEC complex structures, the extremity of the anticodon loop does not contact the synthetase and is poorly disordered, as previously observed for the T. thermophilus system.

We obtained structures of E. coli LeuRS with the tRNA in the editing conformation either in the absence of other substrates, or with leucine or LeuAMS in the synthetic site, or with the benzoxaborole covalent adduct in the editing site. In the absence of benzoxaborole, the 3′ CCA end of the tRNA is less tightly bound in the editing active site, and the last four base pairs of the acceptor stem and discriminator base, which are not in contact with protein, are poorly ordered owing to flexibility. Formation of the benzoxaborole-tRNA adduct strongly stabilizes tRNA binding in the editing site and improves the ordering of the tRNA acceptor stem, although the mobility (as judged by the crystallographic B-factors) of the last two base pairs and discriminator base is still high. The interactions of the 1-hydroxy-3H-2,1-benzoxaborole in the editing active site are identical to those previously described for similar compounds. Binding of leucine or LeuAMS results in little change of the structure apart from the closure of Tyr43, which is otherwise in an open configuration, over the substrate leucine (see earlier).

Global comparison of the editing and aminoacylation states
Both the tRNA and the flexibly linked domains markedly change their orientation during the transition between the aminoacylation state to the editing state (Fig. 3a, b and Supplementary Video 1). A major feature of the aminoacylation complex compared to the editing state
conformation is that the entire leucine-specific domain rotates 33° toward the synthetic active site. This brings the β-hairpin 577–583 into contact with the ZN1 domain, at the same time breaking the contact that occurs in the editing state between the 595–601 hairpin and the tRNA. The entire tRNA pivots by 15° around conserved contacts with the anticodon-binding domain (Fig. 3a), tipping it toward the synthetic active site. The pivoting tRNA is accompanied by the C-terminal domain, maintaining its保守 interactions with the T-loop and long variable arm (Fig. 2d and Supplementary Fig. 2c). The editing domain rotates by 12° to open up a passage for translocation of the 3′ end of the tRNA from the otherwise closed aminoacylation state. Although in the E. coli system the ZN1 domain is ordered in only the aminoacylation state, in structures of LeuRSTT with bound adenylate (PDB 1H3N) or with tRNA in the editing state (PDB 2BYT), the ZN1 domain is packed over the leucyl-adenylate in a position completely incompatible with tRNA 3′-end binding (Supplementary Fig. 3c). This position may help to prevent premature hydrolysis of the adenylate, but the domain then needs to rotate by about 44° into a position that is compatible with 3′-end binding (Supplementary Fig. 3d,e). More detailed implications of these domain motions are discussed later.

**Active site interactions of the KMSKS loop**

Comparison of the E. coli LeuRS aminoacylation and editing complexes shows that the class I conserved and catalytically important 619-KMSKS loop moves as an integral part of the leucine-specific domain. The movement brings it some 6–7 Å closer into the active site in the aminoacylation state, allowing it to make crucial interactions with the 3′ strand that correctly position the tRNA extremity for the transfer reaction (Figs. 2a and 4a,b). In contrast, in the editing conformation, there are no contacts to the 3′ strand of the acceptor stem (Supplementary Fig. 1b). In the closed aminoacylation state, Met568 flips to stack against the center of the adenine base of the LeuAMS (Fig. 4a). This correlates with a rotamer switch of Glu532, allowing it, the N3 of A76 and the LeuAMS sulfate to coordinate a water molecule, or possibly a magnesium ion, that could be important in the catalytic mechanism (Fig. 4b,c, discussed later). One result of this tightening of the active site is that the adenylate itself is slightly compressed into a strained configuration in comparison to the more extended conformation observed in structures without the tRNA in the aminoacylation state (Fig. 4a,b). Whereas the leucyl moiety is unchanged, the adenylate ribose is closer to the carbonyl group, partly owing to the maintenance of the interaction of its 2′-OH with Glu532, which, as mentioned, has changed side chain conformation (Fig. 4b). In contrast, in the editing conformation with leucine or LeuAMS in the synthetic site, the KMSKS loop is in an open, relaxed conformation (Fig. 4a). In this case, the adenine moiety of the LeuAMS is much less tightly bound, with no base-specific contacts to the backbone of Val569 and Met620 and no stacking with Met568 (Fig. 4a,b).

These observations are consistent with a previous suggestion based on TyrRS structures that the KMSKS loop could be in three states: open, semi-open and closed32. With the tRNA in the editing conformation, and either without synthetic site substrates or with bound leucine or LeuAMS, the KMSKS loop is in the fully open conformation and unable to form base-specific interactions to the adenine (Fig. 4a). With adenylate bound in the absence of tRNA (LeuRSTT–LeuAMS complex12; PDB 1H3N), the KMSKS loop is in a semi-open conformation with base-specific interactions to the adenine base (Fig. 4a). The fully closed conformation of the KMSKS loop has been observed only with the leucyl-adenylate bound together with the 3′ of the tRNA (Fig. 4b). However, a similar state might occur for the pre-activation state when ATP and leucine are both bound, as observed, for instance, for TyrRS19 (PDB 1H3E) or GlnRS14. Nonetheless, the only known structure of a class I enzyme with an ATP analog bound, but no amino acid substrate—that of P. horikoshii ArgRS24 (PDB 2ZUE)—has the KMSKS loop in the semi-open state. As suggested for the tyrosyl system, the semi-open post-activation state with bound LeuAMP (as observed with LeuRSTT; Fig. 4a) may be required to permit initial binding of the tRNA 3′ end before reclosure, induced by 3′-end interactions, to allow the aminoacylation reaction.
The crucial role of the KMSKS loop in activation and aminoacylation, and its coupling with the leucine-specific domain explain why a gross deletion of this domain (569–618 replaced by three or five alanines) abolishes aminoacylation activity of E. coli LeuRS. However, the leucine-specific domain is highly variable in size and sequence, and some bacterial LeuRS lack it completely: for example, those of Campylobacter jejuni and Helicobacter pylori, and the second LeuRS (denoted B2) from Agrobacterium radiobacter, which is resistant to the natural antibiotic TM84 (an analog of LeuAMP)34. Notably, Mycoplasma mobile has evolved a minimal LeuRS that lacks both the leucine-specific domain and also, uniquely, the entire editing domain35. It remains to be seen how the absence of the leucine-specific domain affects the function of these bacterial enzymes, but one might hypothesize that the KMSKS loop is more dynamic, as it does not have the inertial mass of the rest of the leucine-specific domain to carry with it.

Aminoacylation catalytic mechanism

For class I synthetases, the aminoacylation reaction requires the nucleophilic attack of A76 ribose O2' on the carbonyl carbon of the aminoacyl-adenylate. The reaction is most likely substrate assisted, with the phosphate of the adenylate acting as the general base to abstract the proton from the 2'-OH in a concerted mechanism14,36 (Fig. 4c). This mechanism is potentially universal, as it does not depend on nearby, nonconserved residues, as proposed by some authors15. The LeuRSEC aminoacylation complex structure is generally consistent with this mechanism as the O2' is 3.6 Å from the sulfate oxygen of the leucyl-adenylate analog LeuAMS, which is comparable to 3.8 Å for the equivalent distance in the GlnRS ternary complex (PDB 1QTQ). However, these distances are substantially longer than a hydrogen bond, suggesting that the energy barrier for deproteination might be too high for the direct reaction. This could reflect the fact that the structures do not correspond precisely to the real situation (for example, the sulfamoyl analog lacks the negative charge of leucyl-adenylate) or that additional elements might be required to explain the observed low energy barrier for the aminoacylation reaction.

One fully conserved LeuRS residue that could have a role in the aminoacylation reaction is Glu532, which is strategically placed in the aminoacylation complex (Fig. 4b,c). To test the role of this residue, we expressed an E532Q mutant of LeuRSEC and measured the temperature dependence of the pyrophosphate (PPi) exchange and overall leucylation reactions (Supplementary Fig. 4c). The E532Q mutant had similar activation energies for the first step and the overall reaction (93 kJ mol⁻¹ and 94.9 kJ mol⁻¹, respectively) in contrast to the wild type, which had corresponding values of 25.2 kJ mol⁻¹ and 55.0 kJ mol⁻¹. This suggests that the rate-limiting step for the mutant E532Q has shifted to the ATP-dependent activation of leucine (Supplementary Fig. 4d), consistent with an important role for Glu532 in activation, perhaps by coordinating a magnesium ion. Indeed a water molecule (Wat A2074) that is coordinated by Glu532 could occupy the magnesium position (Fig. 4b,c). In the presence of the authentic, negatively charged LeuAMP, it is plausible that this site could still bind a magnesium ion, and this might facilitate the aminoacylation reaction, even though the E532Q mutant does not show this. Combined quantum-molecular dynamics simulations are likely to be required to determine the most plausible mechanism for the aminoacylation reaction in LeuRS.

DISCUSSION

In the aminoacylation state, the tRNA 3' end is deeply buried in the catalytic domain. How does aminoacyl transfer with concomitant formation of AMP lead to product release and translocation? With reference to the schematic diagram of the two functional states (Fig. 5a,b), we propose the following mechanism for the functional cycle of E. coli LeuRS. We hypothesize that the aminoacylation state is under strain with regard to the unusual conformation of the 3' end of the tRNA, the distorted state of the adenylate and the fully closed conformation of the KMSKS loop. Formation of this state is possible because of the presumably high binding energy of the tRNA 3' end. Once the covalent integrity of the adenylate is broken by completion of the aminoacylation reaction, the strain is released by relaxation of the KMSKS loop into its open conformation. This is accompanied by the dissociation of AMP and the flipping of the entire leucine-specific domain, whose inertia will prevent easy reversion to the closed state. Concomitant with this, the KMSKS loop interactions with the tRNA are broken, destabilizing the binding of the entire acceptor end of tRNA.
the now charged tRNA. This leads to broader cooperative destabilization of the interactions holding the ZN1 domain and the editing domain in contact with each other and with the tRNA. The release of constraints allows the tRNA 3′ end to relax to its preferred conformation, with the discriminator base stacked on the 1:72 base pair and the CCA end now able to enter the editing active site. Locally, this is accompanied by a rearrangement of the interactions of Glu292, Arg416, Arg418 and Tyr330 with the tRNA, each of which has distinct roles in the two states (Fig. 5c, d). More globally, the editing domain rotates slightly as the tRNA pivots around its fixed contact points on the anticodon-binding domain, and the C-terminal domain moves synchronously, maintaining its contacts to the tRNA. In this conformation, the tRNA is already partially dissociated from the catalytic domain (Supplementary Fig. 1a, b and Supplementary Table 1), favoring total tRNA release after proofreading in the case of correctly charged Leu-tRNALeu16–24, or possibly direct re-aminoacylation, without full release, of hydrolyzed mischarged tRNA.

Our structures provide detailed information on the end points of translocation; it might be difficult to obtain crystal structures of eventual intermediates, although an indication of such states was observed for the archaean LeuRSphi aminoacylation complex11. Molecular dynamics simulations can potentially fill in the gap, as used in the analysis of the mechanism for Glu-tRNAGlu release from the synthetic site of class 1b GluRS18. In another study, normal-mode analysis of LeuSTT revealed both correlated and anticorrelated motions between various structural elements35. Correlated motions included the coupling of the KMSKS loop with the leucine-specific domain, and of the editing domain loop 286–298 with the ZN1 domain; anti-correlated motions were detected between the leucine-specific and editing domains, consistent with the structure-based results described here. In the future, a completed ensemble of structures (including the currently lacking preactivation complex with bound ATP) together with molecular dynamics simulations and fast kinetic studies coupled with structure-based mutagenesis will hopefully provide further insight into the dynamics of the functional cycle of bacterial leucyl-tRNA synthetase. Notably, recent results have revealed that eukaryotic, cytoplasmic LeuRS acts as an intracellular leucine sensor in the amino acid–induced mTORC1 signaling pathway, which regulates cell growth36,39. It is proposed that Rap GTPases, which activate mTORC1, bind to the LeuRS editing domain and sense the presence of bound leucine via the conformational state of the enzyme. This particularly exemplifies the need for further analysis and understanding of the conformational dynamics of LeuRS.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Protein Data Bank: Atomic coordinates and structure factors for the editing complex with l-leucine have been deposited with accession code 4ARC; for the orthorhombic and monoclinic forms of the complexes with benzoazoborole under accession codes 4ARI and 4AS1, respectively; and for the aminoacylation complex with accession code 4AQ7.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

T.C. crystallized the editing-state complexes and A.P. crystallized the aminoacylation complex. T.C., A.P. and S.C. collected X-ray data and performed the structural analysis. M.T.V. and T.L.L. performed the mutagenesis and associated biochemical studies under the supervision of S.A.M. S.C. wrote the manuscript with input from all other authors. S.A.M. acknowledges funding from US National Institutes of Health grant GM63789.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Crystallization. E. coli LeuRS with an N-terminal six-histidine tag and E. coli tRNA<sub>Lua</sub> transcript were prepared and purified as described<sup>40,41</sup>. Protein was stored in buffer comprising 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM MgCl<sub>2</sub> and 5 mM 2-β-mercaptoethanol. Crystallization was performed at 20 °C by the hanging drop vapor diffusion method. For the editing complex, solutions were prepared with 33 μM LeuRS, 40 μM tRNA<sub>Leu</sub> and 1 mM l-leucine or 1 mM 1-hydroxy-3,4,2-benzoxaborole (a gift from Anacor Pharmaceuticals). Cocrystals were obtained by mixing 2 μl of this solution with 1 μl of reservoir solution containing 0.1 M sodium acetate (pH 5.5), 14–18% (w/v) PEG 6000 and 200 mM NaCl. The crystals were frozen in liquid nitrogen for a few seconds in the mother liquor, which contained 22% (v/v) ethylene glycol as cryoprotectant and the corresponding small substrates. In the case of the aminoacylation complex, solutions were prepared with 33 μM LeuRS, 50 μM tRNA<sub>Leu</sub> and 1 mM leucyl-adenylate analog (LeuAMS, purchased from RNA-TEC). Cocrystals were obtained by mixing 2 μl of this solution with 2 μl of reservoir solution containing 0.1 M bis-TRIS (pH 5.5), 23–25% (w/v) PEG 3350 and 200 mM ammonium acetate. The crystals were frozen in liquid nitrogen before X-ray exposure without added cryoprotectant.

Structure determination and refinement. All diffraction data sets were collected at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) using a wavelength of 1.072 Å for the complex with l-leucine, 0.931 Å and 0.934 Å for the orthorhombic- and monoclinic-crystal forms, respectively, of the complex with benzoxaborole and 0.938 Å for the complex with LeuAMS. Data were integrated and scaled with the XDS suite<sup>42</sup>. Further data analysis was performed with the CCP4 suite<sup>43</sup>. The structure of the LeuRS–tRNA<sub>Leu</sub>–l-leucine editing complex was initially solved by molecular replacement with Phaser<sup>44</sup> using as models the <i>T. thermophilus</i> LeuRS (structure PDB 1H3N)<sup>45</sup> without its editing domain and the E. coli LeuRS editing domain structure (PDB 2AIG)<sup>46</sup>. The model was improved by automatic building using ARP-WARP<sup>47</sup>, and manual adjustments were made with COOT<sup>48</sup>. The structure of the aminoacylation complex was solved by molecular replacement with Phaser using the core of the protein from another structure (PDB 3G55) as a guide. All models were refined using REFMAC5 with TLS. The orthorhombic (P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>) and monoclinic (P2<sub>1</sub>) forms of the LeuSEC editing complex have only minor differences involving a small coordinated rotation of the C-terminal domain tRNA and editing domain, without changing specific protein–tRNA interactions. In the monoclinic form, the C-terminal domain is partially disordered, and in general the B-factors are slightly higher. Interfaces were analyzed with the PISA server (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html) and domain motions with the program DynDom<sup>49</sup>. Structure quality was analyzed with MolProbity<sup>50</sup> (http://molprobity.biochem.duke.edu/) and showed all residues in allowed regions (with 95.1–98.0% of residues in favored regions) for the different models. Figures were drawn with PyMOL (http://www.pymol.org/), and a video showing the conformational changes between the aminoacylation and editing states was generated by CHIMERA (http://www.cgl.ucsf.edu/chimera/).

Enzyme assays. A reaction mixture consisting of 60 mM Tris (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM DTT, 4 mM ATP, 21 μM [32P]leucine (10 μCi nmol<sup>-1</sup>) and 2.5 μM tRNA<sub>Leu</sub> was initiated by the addition of 25 nM enzyme and quenched<sup>51</sup>. Higher concentrations of 4 μM tRNA<sub>Leu</sub> and 1 μM enzyme concentrations were used for weak mutant activities (Supplementary Fig. 4b). Leucine-dependent PPI-ATP exchange reactions contained 50 mM HEPES (pH 8.0), 10 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM ATP, 1 mM [32P]PPi, (10 μCi μmol<sup>-1</sup>) and 1 mM leucine and were initiated by 10 nM enzyme. Each reaction was quenched on Baker-Flex polyethyleneimine cellulose TLC plates and chromatographed in 750 mM KH<sub>2</sub>PO<sub>4</sub> (pH 3.5) and 4 M urea at 25 °C.

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