Analysis of the Resistance Mechanism of a Benzoxaborole Inhibitor Reveals Insight into the Leucyl-tRNA Synthetase Editing Mechanism

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Supporting Information

ABSTRACT: A new class of antimicrobial benzoxaborole compounds was identified as a potent inhibitor of leucyl-tRNA synthetase (LeuRS) and therefore of protein synthesis. In a novel mechanism, AN2690 (5-fluoro-1,3-dihydro-1-hydroxy-2,1-benzoxaborole) blocks fungal cytoplasmic LeuRS by covalently trapping tRNALeu in the editing site of the enzyme’s CP1 domain. However, some resistant mutation sites are located outside of the CP1 hydrolytic editing active site. Thus, their mode of action that undermines drug inhibition was not understood. A combination of X-ray crystallography, molecular dynamics, mutadynamics, biochemical experiments, and mutational analysis of a distal benzoxaborole-resistant mutant uncovered a eukaryote-specific tyrosine “switch” that is critical to tRNA-dependent post-transfer editing. The tyrosine “switch” has three states that shift between interactions with a lysine and the 3′-hydroxyl of the tRNA terminus, to inhibit or promote post-transfer editing. The oxaborole’s mechanism of action capitalizes upon one of these editing active site states. This tunable editing mechanism in eukaryotic and archaeal LeuRSs is proposed to facilitate precise quality control of aminoacylation fidelity. These mechanistic distinctions could also be capitalized upon for development of the benzoxaboroles as a broad spectrum antibacterial.

The discovery of the antifungal AN2690 (5-fluoro-1,3-dihydro-1-hydroxy-2,1-benzoxaborole) and its novel mechanism of action launched a new frontier for antimicrobial development that is centered on the oxaboroles. The boron-containing compounds bind to a secondary active site in leucyl-tRNA synthetase (LeuRS), which is located in the protein synthesis enzyme’s CP1 domain and is responsible for amino acid editing. When the terminal ribose at the 3′ end of tRNA binds to this hydrolytic site, the boron forms an adduct with the ribose cis-diols to cross-link tRNA substrate and inactivate the enzyme. The inactivated LeuRS−substrate complex cannot turn over to carry out its aminoacylation activity that is critical to cell maintenance and survival.

LeuRS is a member of the essential family of aminoacyl-tRNA synthetases (aaRSs). Each aaRS is specific for a single standard amino acid. In a canonical aminoacylation active site, the enzyme activates amino acid in an ATP-dependent mechanism that forms aminoacyl-adenylate. The amino acid is then transferred to tRNA via an aminoacyl link to either the 2′ or 3′ hydroxyl of the terminal ribose (Figure 1a). In about half of the aaRSs, a hydrolytic site for quality control is located in a separate domain to clear misactivated amino acids in order to circumvent translation of statistical errors during protein synthesis (Figure 1b).

The aaRSs were first used as a clinical target for antibiotics when a natural product pseudomonic acid was discovered to inhibit isoleucyl-tRNA synthetase (IleRS) and subsequently marketed as mupirocin to treat topical Staphylococcus aureus infections. Pseudomonic acid interacts with the synthetic active site to inhibit amino acid activation. In contrast, AN2690 binds to the secondary hydrolytic site and inactivates aminoacylation by forming an adduct with the tRNA−enzyme complex (Figure 1c). This novel antifungal was recently approved as a new therapeutic called Kerydin to treat...
Distal Lys510 Is a Resistance Site for Oxaborole Inhibition of Candida albicans LeuRS. Spontaneous mutations in the yeast cytoplasmic LeuRS CP1 editing domain were isolated in varied concentrations of AN2690.1,13 These mutations lacked resistance to other common antifungal drugs supporting a unique mechanism of action for the oxaborole family of inhibitors.13 Comparison to crystal structures of LeuRS bound to AN2690 showed that the LeuRS editing site was targeted for resistance.1 In addition, a conserved lysine residue outside of the editing site in yeast LeuRS was selected. Since this site is distal to the editing site (Figure 1d), its mechanism of action for resistance to AN2690 (64-fold increased resistance in vivo) as well as a putative role in amino acid editing were unclear. Because this lysine is found across the structure14 and primary sequence alignments of eukaryotic cytoplasmic and archaeal LeuRSs (Figure 1e), we hypothesized that it could play a role in post-transfer editing.

Using a cloned LeuRS gene from the pathogen C. albicans, we introduced the resistance mutation (K510E) at the conserved lysine resistance site. In addition, we substituted an alanine (K510A) to probe the oxaborole mechanism of action and the enzyme target’s resistance mechanism. The wild-type C. albicans LeuRS and each mutant and their CP1 domains were expressed recombinantly in Escherichia coli with an N-terminal six-histidine fusion and purified by nickel affinity chromatography. Circular dichroism of the CP1 domains of the wild type and mutant LeuRS proteins supported that the mutations did not impact the overall structures (Figure S1).

We also cloned the gene for C. albicans tRNA^{Leu}_{eu}, that would be critical to the oxaborole inhibition mechanism. We introduced a T7 promoter and in vitro transcribed tRNA^{Leu}_{eu} using T7 RNA polymerase.15 Purified C. albicans tRNA^{Leu}_{eu} and LeuRS wild-type or mutant protein were combined in leucylation assays. Each mutant LeuRS aminocacylated leucine similarly to the wild-type LeuRS (Figure 2a, Table S1). A concentration of 0.5 mM AN2690 was preincubated with the reaction mixture, and [14C]-Leu-tRNA^{Leu}_{eu} was measured at different time points. Incorporation of the K510A and K510E mutations into the pathogen C. albicans LeuRS conferred resistance to AN2690 in LeuRS aminocacylation assays (Figure 2b).

Because AN2690 targets the editing active site of LeuRS, we also tested for quality control by the C. albicans enzyme. As would be expected, the wild-type C. albicans LeuRS readily deacylated mischarged C. albicans Ile-tRNA^{Leu}_{eu} (Figure 2c). Although the initial velocity of the deacylation activity for the K510E resistance mutation was decreased compared to wild-type, it retained significant activity, supporting that it could maintain quality control of translation at some level in the pathogen. In contrast, the editing activity of the K510A mutant LeuRS was dramatically reduced. We also created a K510R and K510D mutation to test mutations that would be respectively homologous at these sites for the wild-type and resistant eukaryote cytoplasmic and archaeal LeuRSs.

RESULTS AND DISCUSSION

Figure 1. LeuRS CP1 editing domain targeted for inactivation of tRNA^{Leu}_{eu}-dependent oxaborole mechanism of action. (a) LeuRS two-step aminocacylation and hydrolytic editing reactions. (b) Aminoacylation (PDB ID: 4AQ7) and editing complexes10 (PDB ID: 4ARC) of tRNA^{Leu}_{eu}-bound (blue) E. coli LeuRS. The CP1 domain is highlighted in cyan. (c) Antifungal AN2690 and AN3018 react with cis-diolis of AMP to form mimic of tRNA^{Leu}_{eu}-drug adduct. (d) Crystal structure of C. albicans cytoplasmic LeuRS with AN3018-AMP bound14 (PDB ID: 2WFG) and Lys510 resistance site highlighted. (e) Sequence alignment of eukaryotic and archaeal LeuRS with Lys510 marked by an arrow. Blue highlights the resistance site, while yellow reflects >50% homology in the sequence shown.

ONYCHOMYCOSIS. A novel benzoxaborole has also demonstrated efficacy in combatting complicated infections caused by some Gram-negative bacteria.12

Resistance mutations to AN2690 have been identified in Candida albicans.1 Most are located in the hydrolytic active site of the CP1 domain. However, some are distal to the catalytic site, and their resistance mechanisms are unclear. For example, mutation of a conserved lysine residue (K510E) was selected at least 9 Å away from the bound drug molecule (Figure 1d). We hypothesized that the distally located Lys510 plays a role in amino acid editing within the CP1 domain. Mutational analysis of this region combined with X-ray crystallography, molecular dynamics (MD), and metadynamics simulations as well as biochemical experiments uncovered a three-state tyrosine switch in the editing mechanism that the AN2690 capitalized upon to cross-link the enzyme-bound tRNA. It also implicated an evolutionary partition that distinguished archaeal and eukaryotic mechanisms for editing from bacteria.
LeuRS. The K510R LeuRS mutant retained deacylation activity compared to the wild-type, while the K510D mutation activity was even less than the selected K510E resistance mutant. We hypothesized that this drug resistance mutant had selected a critical editing site, which balanced combatting AN2690, while maintaining sufficient quality control.

**K510A LeuRS Mutation Traps a Critical Tyrosine in an Oxaborole-Resistant Rotamer State.** The co-crystal structure of the *C. albicans* LeuRS CP1 domain bound to AN3018-AMP has been solved previously (Figures 1c, 3a; PDB IDs: 2WFE, 2WFG). The oxaborole resides largely in the amino acid binding pocket of the editing active site, while the AMP mimics the 3′ terminus of the tRNA that forms a dative bond with the boron atom. Here, we co-crystallized the wild-type *C. albicans* LeuRS CP1 editing domain with AN2690-AMP and determined the structure of the complex at 2.0 Å resolution (Figure 3b, Table S2). In both of the oxaborole-bound structures that represent the LeuRS–inhibitor complex (I-state), the hydroxyl group of the Tyr487 ring binds to the phosphate group of the AMP adduct. As observed previously, there is also a second rotamer form, where Tyr487 interacts with Lys510.14

Tyr487 resides in the I4ae α-helix that “caps” the editing active site of *C. albicans* LeuRS by closing over it. In yeast, a separate mutation that confers resistance, in which an aspartate was substituted by either a glycine or asparagine, was proposed to disrupt a hinging mechanism for the cap.13 This α-helix cap is comprised of a peptide insert that is found only in eukaryotes and archaea and missing in bacterial LeuRSs (Figure S2).17,18 Comparison with the structure of the apo CP1 domain from *C. albicans* LeuRS (Figure 3a) showed that the α-helix cap moves 1.5 Å toward the oxaborole upon its binding to the editing site.14 Concomitantly, the conserved Tyr487 transitions between two rotamer states to interact with the inhibitor in the I-state (Figure 3a). Crystal structures of LeuRS from humans as well as *Pyrococcus horikoshii* also support that the tyrosine has two rotamer conformations (Figure S2).

In the absence of the oxaborole-AMP adduct, Tyr487 interacts electrostatically with Lys510 (K-state; Figure 3a). A nearby phenylalanine (Phe491) stabilizes the tyrosine via π–π interactions. Because Lys510 is an oxaborole resistance site, we hypothesized that the Tyr487–Lys510 interaction played a critical role in the mechanism of resistance. We determined that the $K_D$ for binding of AN2690-AMP to the *C. albicans* LeuRS CP1 domain was increased significantly from 0.7 ± 0.3 μM for the wild-type enzyme to 21 ± 3 μM and 46 ± 6 μM for the K510E and K510A mutant LeuRSs, respectively (Table S3).

**Figure 2.** Lys510 mutants of *C. albicans* LeuRS confer tRNA<sub>Lue</sub>-dependent AN2690 resistance. Enzyme assays were carried out as described previously. (a) Leucylation activity. (b) AN2690 inhibition of *C. albicans* LeuRS aminoacylation. Open symbols indicate presence of AN2690. (c) LeuRS deacylation of Ile-tRNA<sub>Lue</sub>. Error bars are based on reactions repeated at least in triplicate.

**Figure 3.** Structure and thermodynamics of oxaborole-AMP drug binding to *C. albicans* LeuRS CP1 domain. Crystal structure superposition of apo wild-type LeuRS CP1 domain (PDB ID: 2WFE) binding to AN3018-AMP (a; PDB ID: 2WFG) or AN2690-AMP (b; PDB ID: 5AGJ). (c) Comparison of apo K510A mutant LeuRS CP1 domain (PDB ID: 5AGH) and binding to AN2690-AMP (PDB ID: 5AGI). Drug-bound and apo LeuRS are yellow and cyan, respectively. AN3018-AMP, AN2690-AMP, Tyr487, and Lys510 are in “licorice” format. Red arrows mark the movement (1.5 or 0.9 Å) of the I4ae helix, which was measured by the change in distance of the α-carbon of the Val477 in the overlapped structures.
Isothermal titration calorimetry indicated that the change in binding was largely driven by entropic factors (Figures 4, S3). We wondered if the entropy loss correlated to a restriction in the tyrosine rotamer states.

Figure 4. ITC-derived thermodynamic parameters obtained for the binding of AN2690-AMP to wild-type C. albicans LeuRS and mutants. Error bars represent the standard deviation (<5%) of at least two independent experiments.

We screened for crystallization conditions for the Lys510 mutants of LeuRS in the presence and absence of the oxaborole inhibitor. Both the apo and AN2690-AMP bound forms of the K510A C. albicans CP1 domain crystallized at 1.8 and 1.5 Å, respectively, via hanging drops in an overnight incubation at 16 °C (Table S2). In both apo and AN2690-AMP bound forms, the side chain of Tyr487 is locked into the “K-state” by occupying space vacated by the lysine side chain in the mutant Lys510A LeuRS (Figure 3c). Tyr487’s failure to form a hydrogen bond with AN2690-AMP in conjunction with restricted movement of the α-helix in which it resides is consistent with its weaker K_d for drug binding.

An Ile-AMP analog of the mischarged tRNA molecule was built (Figure 5b) and modeled into the editing site based on structural overlap between C. albicans LeuRS (PDB ID: 2WFG) and T. thermophilis LeuRS that is bound to the mischarged norvaline-tRNA (PDB ID: 2BTE). This bound Ile-AMP overlapped with the AN2690 inhibitor complex in having a hydrogen bond bridge between its phosphate moiety and Tyr487. We probed the rotamer transitions of Tyr487 using MD via NAMD. In four sets of simulations for the C. albicans LeuRS, Tyr487 transiently maintained its interaction with the phosphate group of Ile-AMP that mimics the bound oxaborole-AMP of the I state for ~10 ns. Over the combined 60 ns time course of MD for the wild-type LeuRS CP1 domain, the K-state rotamer was sampled where Tyr487 interacted with Lys510 and Phe491 similar to the static crystal structure of apo LeuRS. An additional rotamer state called “S” emerged in the presence of the Ile-AMP editing substrate where Tyr487 forms a hydrogen bond with the 3′ hydroxyl group of the ribose of the editing substrate. In the mutant K510E LeuRS, MD only showed the K-state and S-state, even though Lys510 has been substituted by a glutamate residue. Similar to the I-state that binds the oxaborole-AMP inhibitor and transiently interacts with the phosphate moiety of Ile-AMP, the 4Iae α-helix shifts closer toward the editing pocket (Figure 5a,d).

Figure 5. MD and WT-meta computed states of C. albicans LeuRS drug target. (a) MD of C. albicans LeuRS CP1 domain (PDB: 2WFG) initially docked to Ile-AMP yields three states in which Tyr487 interacts with the ribose 3′ hydroxyl group (“S,” left), phosphate (“I,” middle), and the Lys510 resistance site as well as Phe491 (“K,” right). (b) Ile-AMP substrate mimic used in simulations. (c) Sequence alignment of eukaryotic and archaeal LeuRS with Tyr487 highlighted by an arrow. (d) Four independent standard MD simulations sampled three conformational states of wild-type (left) and K510E resistance mutant (right) of C. albicans LeuRS CP1 domain based on distances between the Tyr487 hydroxyl group and 3′ hydroxyl group of Ile-AMP ribose. The “S,” “I,” and “K” states are defined respectively by distances of ~3, ~5, and ~9 Å. (e) Free energy map of wild-type (top; simulation time of 200 ns) and K510E (bottom; simulation time of 160 ns) CP1 domain with Ile-AMP bound at 120 ns indicates a relationship of “S,” “I,” and “K” states. The x and y axes represent CV_1 and CV_2 as defined in the text.
We compared the free energy landscape of wild-type and KS10E mutant LeuRS CP1 domains by employing well-tempered metadynamics\(^{11,22}\) (WTmeta) along two collective variables (CVs). The first CV was the distance between the Tyr487 hydroxyl group of the \(C.\) \(albicans\) LeuRS CP1 domain and the 3′-hydroxyl group of substrate Ile-AMP ribose. The second CV was the root-mean-square deviation (RMSD) of the side chain atoms of Tyr487 and the backbone atoms of the shifting 4Iae \(\alpha\)-helix, where Tyr487 resides. After reaching convergence for both simulations (Figure 5e, S4a,c), we compared the free energy difference (\(\Delta G\)) for the K-state and I-state. For the KS10E LeuRS mutant, the free energy difference is stabilized by \(\sim 3\) kcal/mol with respect to the wild-type (Figure 5b). This difference correlates well with the ITC measurements described above that determined a 2.0 kcal/mol difference in the \(\Delta G\) of binding (Table S3).

Collaboration of Amino Acid Neighbors for LeuRS Post-transfer Editing Diverge between Bacteria and Eukaryotes. Our computational investigations implied that Tyr487 might play a mechanistic role in post-transfer editing through its interaction with the ribose 3′ hydroxyl group (Figure 6a). This tyrosine is conserved in eukaryotes but located in a peptide insert (I4ae) that is completely missing in bacteria (Figures 5c, S2).\(^{14}\) In contrast, a bacterial-conserved threonine residue (Thr248 in \(E.\) \(coli\) and \(T.\) \(thermophilus\)) in a completely different region of the CP1 domain interacts with the 3′ ribose hydroxyl (Figure 6b).\(^{18,19,23}\) Remarkably, this threonine resides in a peptide region that is conserved among all three kingdoms but is only capitalized upon by bacteria for interactions with the substrate’s hydroxyl group. This threonine-rich peptide is critical for substrate binding, discrimination, and hydrolysis during the editing reaction.\(^{24}\)

However, eukaryotes have substituted this threonine (Thr248) in this otherwise conserved peptide with leucine (Figure 6c).

On the basis of experimental\(^{24}\) and computational QM/MM investigations,\(^{23,25}\) Thr247 along with a neighboring threonine (Thr248) within the highly conserved threonine-rich region of the bacterial LeuRS CP1 editing domain were hypothesized to collaborate to stabilize the transition state during ester bond cleavage of misacylated tRNA\(^{24}\) (Figure 6b). The first threonine (Thr247) interacts via a hydrogen bond with the carbonyl oxygen atom of the aminoacyl group of mischarged tRNA. This carbonyl oxygen also forms a hydrogen bond with a nearby water molecule in a bipartite interaction.\(^{23,25}\)

As described above, the neighboring threonine (Thr248) interacts with the 3′ hydroxyl group of the terminal adenine ribose of tRNA (Figure 6b). These neighboring bacterial threonines sustain a network of hydrogen bonds that stabilize a high-energy state in the hydrolytic mechanism.\(^{25}\)

Individual mutation of each bacterial threonine residue had minimal effects on decylation activity, but a double mutation of the threonine neighbors to valine abolished hydrolytic activity of LeuRS.\(^{24}\)

We performed a 30 ns MD simulation using the bacterial \(T.\) \(thermophilus\) LeuRS CP1 domain bound to Ile-AMP. Thr248 predominantly interacts with the 3′ hydroxyl group of the tRNA terminal adenine ribose (Figure S5a), while the interaction between Thr247 and the substrate’s carbonyl oxygen was frequently sampled (Figure S5b).

Significantly, MD simulations that we describe above for the S state suggest that Tyr487 of \(C.\) \(albicans\) LeuRS mimics the role of the second “threonine collaborator” in bacteria LeuRSs that is missing in eukaryotes. Tyr487 forms a hydrogen bond to the 3′ ribose hydroxyl but approaches the bound editing substrate mimic from its opposite side compared to the bacterial threonine (Figure 6a). This is because Tyr487 resides in the eukaryote-specific I4ae peptide insert that is distant from the conserved threonine-rich peptide. Of the Tyr487 substitutions that selectively introduced alanine, phenylalanine,
or glutamic acid, only the Y487E mutant exhibited small effects on deacylation (Figure 6d), reminiscent of the minor effect when the downstream threonine neighbor was singularly mutated in bacterial LeuRS.24

Primary sequence alignments indicated that the downstream threonine neighbor in bacteria has been replaced by leucine in eukaryotes (Figure 6c). To identify hydroxyl-bearing candidates that could serve as a putative collaborative residue with tyrosine, we compared the three-dimensional structures of the CP1 domain editing sites. We hypothesized that Thr316 of C. albicans LeuRS structurally overlaps with the first threonine neighbor (Thr247) of T. thermophilus LeuRS for deacylation (Figure 6a) and could collaborate with Tyr487. Significantly, Thr316 is also aligned in the primary sequence with the critical threonine neighbor in bacteria (Figure 6c). Our MD simulation result also supported that Thr316 interacted with the Ile-AMP carbonyl group (Figure S5c).

Similar to the Tyr487 mutations in C. albicans LeuRS, single mutations of Thr316 to valine and serine only had minimal effects on deacylation (Figure 6e). However, a double mutation of Y487F and T316V that removed both hydroxyl groups from these side chains in C. albicans LeuRS abolished editing, consistent with the bacterial LeuRS mechanism.24 This supports that bacteria and eukaryotes have separately adapted to rely on different structural elements to facilitate post-transfer editing while maintaining fidelity. Whereas bacteria utilize two neighboring threonine residues, eukaryotes depend on one threonine and a tyrosine, more distant in the primary protein sequence, to structurally converge in the active site and collaboratively facilitate deacylation.

**Substrate-Assisted Mechanism for LeuRS Post-transfer Editing Relies on 3′ Hydroxyl Groups of Mischarged tRNA.** Computational QM/MM investigations23,25 suggested that, in bacterial LeuRS, the 3′ hydroxyl group of tRNA is important to a network of hydrogen bonds that facilitates hydrolysis of mischarged tRNALeu. If this is the case, then we rationalized that modifications of the hydroxyl group at this site, which hinder its interactions with the collaborative tyrosine, would impact deacylation activity. We used E. coli tRNA nucleotideyltransferase to exchange the terminal adenosine of C. albicans tRNAFeu transcript with a hydrogen (tRNA-3′-H), amine (tRNA-3′-NH2), and methoxy (tRNA-3′-OCH3) group (Figure S6a).26,27 Each of these tRNAs were mischarged with isoleucine and recovered for deacylation assays.

In each case, modification at the 3′ hydroxyl group impeded deacylation activity relative to the wild type mischarged tRNAFeu (Figure 6f). Introduction of an amine (tRNA-3′-NH2) resulted in the most significant decrease in deacylation. However, it is likely that this 3′ amine group attacks the carbonyl carbon of the mischarged isoleucine to promote an intramolecular shift of the amino acid, yielding a stable peptide linkage28–30 at the 3′ position that is not readily hydrolyzed by LeuRS (Figure 6b).

As expected, replacement of the 3′ hydroxyl group with a bulky methoxy group displaces the bound water molecule to substantially decrease deacylation activity. On the basis of initial velocities and plateau levels of deacylation activity, the 3′ deoxyATP replacement at the tRNA 3′ end maintained some activity, albeit decreased relative to the wild-type tRNALeu. This could indicate that a shift of the charged isoleucine to the 3′ position is not a prerequisite for deacylation at the 2′ hydroxyl group. An alternative hypothesis is that similar to substitutions of serine by alanine that replaced a critical hydroxyl group in the active site of the protease subtilisin,31 it is possible that the void resulting from complete elimination of the ribose hydroxyl group could be filled by a water molecule. This water molecule would enable at least limited deacylation by re-establishing the hydrogen bond network to promote hydrolysis.

**Conclusions.** The oxaboroles have emerged as a whole new class of antimicrobials that targets essential LeuRS enzymes via their amino acid editing site. This boron-dependent mechanism is substrate-assisted, requiring the free cis-diol of the tRNA’s 3′ end to trap a tightly bound RNA-protein complex.1 Specificity by LeuRS for the fused oxaborole benzylic ring is likely facilitated by a roomy editing site that evolved specifically to flexibly accommodate multiple noncognate amino acids (such as isoleucine and norvaline) that LeuRS misactivates.32 This contrasts with closely related aaRSs, such as IleRS and ValRS, which edit by a similar mechanism that requires a universally conserved aspartic acid for quality control but singularly target single amino acids that are smaller.6 Thus, design of effective oxaborole inhibitors for other editing aaRSs would likely require a smaller chemical scaffold to confer suitable specificity.

A combination of biochemical, structural, and computational work determined that the LeuRS undergoes subtle conformational shifts in its editing site that profoundly influence the potency of the mechanism of action for the oxaborole. While revealed by a distal resistance mutation, drug interactions are dependent on a mobile tyrosine residue that migrates between three states. The resting state of LeuRS is comprised of mixed populations of the tyrosine states.14 However, it is clear that the conserved tyrosine switch adopts specific conformations with distinct interactions as it cycles through the LeuRS reaction.

The tyrosine switch is proffered by a later evolutionary addition of a helical insert within the CP1 editing domain that is found only in archaeal and eukaryotic LeuRSs. It is completely missing in bacterial LeuRSs (Figure 7). Rather, the editing mechanism for bacterial LeuRSs is dependent on a conserved threonine residue that is also utilized in IleRS and ValRS. Akin to the tyrosine switch, this threonine has been hypothesized to interact with the 3′ hydroxyl of the bound tRNA during editing.17,22

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**Figure 7.** Mechanistic divergence of LeuRS post-transfer editing. T. thermophilus (green; residues 245–248, 335–347) and C. albicans (yellow; 314–317, 410–422) LeuRS CP1 domains are structurally aligned in the modeled substrate-bound state. The eukaryotic I4ae helix insert is purple. Key amino acids for editing of C. albicans and T. thermophilus LeuRS are colored black and red, respectively.
tRNA ribose that plays an important chemical role in the editing mechanism. Remarkably, the bacterial LeuRS threonine and eukaryotic/archaeal LeuRS tyrosine approach the bound tRNA ribose from nearly opposite sides in its editing complex to confer the hydrogen bond to the 3′ hydroxyl. While the first LeuRS-specific oxaborole (AN2690) discovered was a potent antifungal, this mechanistic difference could provide another avenue to exploit the oxaboroles as antibacterials. A second generation of oxaboroles that capitalize upon the threonine interaction in bacterial LeuRSs might likewise be used to inhibit IleRS and ValRS. At least in the case of LeuRS, the mechanistic distinctions of the editing reaction could further enhance species specificity of an antibiotic that treats a spectrum of bacterial diseases.

Methods

Materials. Radiolabeled [1-14C]-leucine and [3H]-isoleucine were obtained from PerkinElmer and Amersham Pharmacia Biotech, respectively. Modified bases, including cordycepin-5′-triphsophate sodium salt (3′-dATP; Sigma), 3′-amino-3′-deoxyadenosine-5′-triphsophate (3′-dN422A-dATP; Biolog Life Science Institute, Bremen, Germany), and 3′-O-methyladenosine-5′-triphosphate (3′-O2690-dATP; TriLink BioTechnologies), were purchased. Plasmid pHZCaWT expressing C. albicans cytoplasmic LeuRS was a gift from Anacor Pharmaceuticals.

Synthesis of tRNA and tRNA Analogs. The gene for C. albicans tRNAUUAwas amplified by PCR using C. albicans genomic DNA and cloned behind the T7 RNA polymerase promoter into the Template preparation of 450 μg of pHZCatRNAUUA. Preparation template of 450 μg of pHZCatRNAUUA, in vitro transcription, and RNA recovery were performed as described.33 Mischarged tRNAs were prepared using an aliquot of 8 μM in vitro transcribed C. albicans RNA1-28 with 60 mM HEPES (pH 7.5), 10 mM MgCl2, 1 mM DTT, and 4 mM ATP at 37 °C for 3 h.33 A Chroma Spin-30 column was used to remove NTPs. The final concentration was determined using an extinction coefficient of 871 000 M−1 cm−1.35

Modified tRNAs were prepared using 5 μM E. coli CCA-adding enzyme and 15 μM in vitro transcribed C. albicans RNA1-28 and 6 mM modified ATP in reactions that contained 20 mM glycine, at pH 9.0, 20 mM MgCl2, and 1 mM Pi, at 37 °C for 4 h. The reaction was phenol-extracted, followed by ethanol precipitation. Modified tRNAs were confirmed by gel electrophoresis and tested for aminoacylation with tRNA-2′-H as a control.

Protein Mutagenesis and Preparation. Plasmid pHZCaLRSWT encoding wild type C. albicans cytoplasmic LeuRS was used as template for PCR-based mutagenesis.33 Plasmid pHZCaY487F was used to introduce a second T316V mutation. Similarly, plasmid pCaCP1WT14 encoding the isolated cytoplasmic LeuRS34 with 60 mM HEPES (pH 7.5), 10 mM MgCl2, 1 mM DTT, and 4 mM ATP at 37 °C for 3 h. A Chroma Spin-30 column was used to remove NTPs. The final concentration was determined using an extinction coefficient of 871 000 M−1 cm−1.35

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Protein Crystalization and Structure Determination. A stock concentration of 34 mg mL−1 LeuRS K510A mutant was stored in 20 mM Tris, at pH 8.0, 100 mM NaCl, and 5% glycerol and loaded onto a pre-equilibrated Superdex 75 column interfaced with an FPLC (AKTA). The concentrations for the isolated CP1 domain and full-length LeuRS were determined using respective extinction coefficients of 28 100 M−1 cm−1 and 144 510 M−1 cm−1, that were calculated by ProtParam.36

Protein crystallization and structure determination. A stock concentration of 34 mg mL−1 LeuRS K510A mutant was stored in 20 mM Tris, at pH 8.0, 100 mM NaCl, and 5% glycerol (v/v). The apo protein was diluted to 15 mg mL−1 in 20 mM Tris and 200 mM NaCl, at pH 7.5, for crystallization. The AN2690-AMP-K510A LeuRS CP1 domain complex was formed using 30 mg mL−1 protein, 7.5 mM AMP, and 1 mM AN2690. Protein crystals were grown in hanging drops of 1 μL protein solution and 1 μL of crystallization solution. The apo K510A LeuRS mutant was crystallized in 0.2 M ammonium acetate, 0.1 M sodium acetate, at pH 4.0, and 24% PEG 3350 overnight at 16 °C. The AN2690-AMP K510A LeuRS CP1 domain complex was crystallized in 0.2 M sodium acetate, 0.1 M HEPES, at pH 7.4, and 30% PEG 3000 overnight at 16 °C. Crystals were frozen in cryoprotectant solution containing 20% glycerol. The AN2690-AMP wild-type LeuRS CP1 domain complex was crystallized as previously described.15 Diffraction data were collected at the ESRF (Grenoble) for wild-type LeuRS CP1/AN2690-AMP complex crystals and at the APS (Argonne National Laboratory) for K510A mutant CP1 apo/AN2690-AMP complex crystals and were integrated with XDS and scaled with SCXALE or with HKL2000,38 respectively. Further data analysis was performed with the CCP4 suite.39 The structure was solved by molecular replacement with PHASER40 using the C. albicans LeuRS editing domain as a model (PDB: 2WFE). Manual adjustments in COOT were used to correct the position of certain residues and to find solvent molecules. The final models were refined using REFMACS. Interfaces were analyzed with the PISA server (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html). Structure quality was analyzed with MOLPROBITY (http://molprobity.biochem.duke.edu/), showing all residues in allowed regions (>97%). Structure depictions were constructed with VMD.28

Well-tempered Metadynamics Simulation (WtMeta). We applied WtMeta to explore the free energy landscape of Tyr rotamer states that have been observed in MD simulations using the version implemented in NAMD 2.9. We performed two sets of simulations on Ile-AMP in complex with wild-type CaCP1 and the K510E mutant form. The CVs in both simulations were the distance between the Tyr487 hydroxyl group of CaCP1 and the 3-hydroxyl group of substrate Ile-AMP ribose and the root-mean-square deviation (RMSD) of the backbone of α helix (sequence 476–489) and side chain atoms of Tyr487. The bias temperature was 2980 K; the initial deposition rate of Gaussians was 0.02 kcal (mol ps)−1, with a width along CV1 of 0.5 Å and along CV2 of 0.25 Å. Quartic walls were applied to limit the sampling region of the CV space. In well-tempered Ile-AMP to the C. albicans LeuRS editing site.
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References


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