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Zinc is the molecular "switch" that controls the catalytic cycle of bacterial leucyl-tRNA synthetase

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ABSTRACT

The *Escherichia coli* (*E. coli*) leucyl-tRNA synthetase (LeuRS) enzyme is part of the aminoacyl-tRNA synthetase (aaRS) family. LeuRS is an essential enzyme that relies on specialized domains to facilitate the aminoacylation reaction. Herein, we have biochemically characterized a specialized zinc-binding domain 1 (ZN-1). We demonstrate that the ZN-1 domain plays a central role in the catalytic cycle of *E. coli* LeuRS. The ZN-1 domain, when associated with Zn^{2+}, assumes a rigid architecture that is stabilized by thiol groups from the residues C159, C176 and C179. When LeuRS is in the aminoacylation complex, these cysteine residues form an equilateral planar triangular configuration with Zn^{2+}, but when LeuRS transitions to the editing conformation, this geometric configuration breaks down. By generating a homology model of LeuRS while in the editing conformation, we conclude that structural changes within the ZN-1 domain play a central role in LeuRS's catalytic cycle. Additionally, we have biochemically shown that C159, C176 and C179 coordinate Zn^{2+} and that this interaction is essential for leucylation to occur, but is not essential for deacylation. Furthermore, calculated K_d values indicate that the wild-type enzyme binds Zn^{2+} to a greater extent than any of the mutant LeuRSs. Lastly, we have shown through secondary structural analysis of our LeuRS enzymes that Zn^{2+} is an architectural cornerstone of the ZN-1 domain and that without its geometric coordination the domain collapses. We believe that future research on the ZN-1 domain may reveal a possible Zn^{2+} dependent translocation mechanism for charged $tRNA^{Leu}$.

1. Introduction

Aminoacyl-tRNA synthetases (aaRSs) are an ancient family of enzymes that have evolved to ensure the fidelity of the first step of protein translation through the faithful production of correctly aminoacylated tRNAs [1]. There are twenty members of the aaRS family—one aaRS per amino acid [2]. The aaRSs are divided into two classes with each consisting of 10 enzymes [2–4]. These two classes have been partitioned according to structural homology and mechanistic similarities of the aminoacylation reaction [2–4]. The aminoacylation reaction is a two-step reaction that begins with the adenylation of an amino acid followed by the aminoacylation of tRNA [5]. Each enzyme within the family of aaRSs catalyzes the aminoacylation reaction. Surprisingly there is considerable diversity in domain insertions, placement and sequence homology within this family of enzymes [6,7]. Additional domain insertions within the core aminoacylation domain, such as the connective polypeptide (CP1) editing domain, were thought to be later evolutionary additions to aaRSs [8,9]. To support this hypothesis, minimal core aminoacylation domains have been constructed that function similar to the wild type enzyme [8–10]. These findings provide evidence that class I and class II synthetases may have evolved from a common ancestor and additionally that the CP1 domain can enhance the activity of the aaRS, but is not an absolute requirement for aminoacylation to occur [8,9,11,12]. Therefore, domain insertions, similar to the CP1 domain, allowed for specialized charging of specific amino acids by each aaRS and allowed aaRSs to develop auxiliary biological functions [8,13,14].

This research effort focuses on a specific domain insertion, which is the zinc-binding domain. Many aaRSs bind zinc within specialized zinc-binding domains. Some zinc-binding domains are known to recognize and interact with tRNA [15,16]. Others are known to help facilitate the aminoacylation reaction [17]. These examples of different aaRS zinc-binding domains highlight the domain's functional diversification.

Zinc-binding domain diversity also exists across speciation lines for leucyl-tRNA synthetase (LeuRS) enzymes. Two zinc-binding domains called ZN-1 and ZN-2 have been found in LeuRS enzymes. Based on the sequence alignment of bacterial, and eukaryote-mitochondrial LeuRSs, the ZN-1 domain is positioned immediately before the CP1

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domain, while ZN-2 divides the aminoacylation domain in approximately half [18]. Certain subsets of LeuRSs are known to bind a zinc atom in both the zinc-binding domains, while other subsets of LeuRSs bind one zinc atom or may not bind zinc at all although they may still maintain the domains [18]. There is also variability within the domains, in that some LeuRSs may only have one zinc-binding domain, which can be either ZN-1 or ZN-2 [18]. The domains also vary in size, ranging from 30 to 60 residues [18]. Due to this diversity within the zinc-binding domains of LeuRSs, we set out to biochemically understand how the zinc-binding domain may affect *Escherichia coli* LeuRS catalysis, which has never been reported until now.

A crystal structure of *E. coli* LeuRS indicates that this enzyme contains a single zinc-binding domain 1 (ZN-1 domain) that binds to a single zinc atom [12]. The crystal structure highlights the importance of the ZN-1 domain in establishing the aminoacylation conformation and stabilizing the Leu-AMP adenylate [12]. However, the crystal structures presented in Palencia et al. paper do not show *E. coli* LeuRS's ZN-1 domain in the editing conformation, but shows *Thermus thermophilus* LeuRS's ZN-1 domain in the editing conformation. Palencia et al. computationally built a partial ZN-1 domain for the *E. coli* LeuRS editing structure using the *T. thermophilus* LeuRS editing structure as a reference, which provided helpful insight, but failed to capture the truly dynamic nature of the ZN-1 domain in its entirety [12]. Therefore, we computationally generated a homology model of *E. coli* LeuRS's ZN-1 domain in the editing conformation to better visualize how the ZN-1 domain's position and structure may change as the enzyme completes its catalytic cycle. We built our homology model using the *T. thermophilus* LeuRS's ZN-1 domain in the editing conformation because the *T. thermophilus* LeuRS structure is the only crystal structure in existence that visualizes the ZN-1 domain in the editing conformation. Building a homology model of any protein requires a reference structure, since *T. thermophilus* shares more than 40% sequence homology with *E. coli* LeuRS it was the most logical choice [19].

With the homology model, we were able to structurally and biochemically piece together the disjointed information that currently has been published concerning the *E. coli* LeuRS ZN-1 domain. We performed alanine-scanning mutagenesis of the ZN-1 domain and biochemically determined that only C159, C176 and C179 are required to chelate Zn^{2+}. Herein, we report the geometric changes that occur within this zinc chelating pocket of the ZN-1 domain as LeuRS transitions between the aminoacylation and editing conformations. We report the novel structural features of this ZN-1 domain and how these features contribute to the domain's inherent thermodynamic instability, which may enable the domain to adopt dramatically different structural states between the aminoacylation and editing conformations. While LeuRS is in the aminoacylation conformation the ZN-1 domain packs against the active site to stabilize Leu-AMP, but once the enzyme transitions to the editing conformation the ZN-1 domain appears to swing away 14° from the aminoacylation active site. We hypothesize that the movement of the ZN-1 domain away from the active site opens a structural path for charged $tRNA^{Leu}$ to effectively translocate to the CP1 domain since the ZN-1 domain packs tightly against the aminoacylation active site while the enzyme is in the aminoacylation conformation. Global structural perturbations due to molecular "hinge" sites within LeuRS in addition to the "sling-shot" movement of the 3′-$tRNA^{Leu}$ acceptor arm [20] may facilitate the movement of the ZN-1 domain away from the aminoacylation active site.

2. Experimental

2.1. Materials

T7 RNA polymerase (Invitrogen, Carlsbad, CA) was used to in vitro transcribe $tRNA^{Leu}$ via run-off transcription [21]. The tRNA product was purified by urea-containing polyacrylamide gel electrophoresis [21] and quantitated based on absorbance at 260 nm using a molar absorption coefficient of 840,700 M^{-1} cm^{-1} [22]. Purified $tRNA^{Leu}$ was denatured at 80 °C for 1 min, followed by an addition of 1 mM $MgCl_2$ and quick-cooled on ice for re-folding.

2.2. Generating mutations

The wild type *E. coli* LeuRS gene is encoded within the plasmid pRWecLeuRS, which was generated by extracting *E. coli* genomic DNA and utilizing PCR to amplify and subsequently clone *leuS* (GeneBank Accession #EG10532) into pET15b (Fisher Scientific, Pittsburg, PA) as previously demonstrated by Härtlen and Madern [23]. To generate pRWecLeuRS we used synthesized oligonucleotides (Integrated DNA Technology (IDT), Coralville, IA). The forward primer 5′-CGCCATATGA TGCAAGAGCAATACCGCCCG-3′ and reverse primer 5′-CGCGGATCCCGG TTGCTGGTCTAACTCCTC-3′, which contain the *NdeI* and *BamHI* restriction sites, respectively. The resulting pRWecLeuRS was used to insert mutations via PCR generating the plasmids: pRWC159A (C159A), pRWC176A (C176A), and pRWC179A (C179A). These mutations were constructed using synthesized complementary oligonucleotides (IDT, Coralville, IA), the forward primers are as follows: C159A, 5′-GCGGTC AACTGGGCTCCGAACGACCAG-3′; C176A, 5′-CGACGGCTGCGCTTGGCG CTGCG-3′; and C179A, 5′-CTGCTGGCGCGCTGATACCAAAG-3′. In addition, we generated the T252Y mutant to purify the mischarged product [^{3}H] Ile-$tRNA^{Leu}$ (as described below) with the following forward primer: 5′-CCCGCCGGACTACTTTATGGGTTGTACC-3′ [24]. DNA sequencing for all plasmids was carried out by Eurofins MWG Operon, Huntsville, AL.

2.3. Protein expression

To express the ZN-1 domain LeuRS mutants, the zinc-binding domain mutant plasmids were used to transform *E. coli* BL21 (Stratagene, La Jolla, CA). A single colony was used to inoculate 5 ml of Luria-Bertani medium containing 100 μg/ml ampicillin (LB-Amp) and grown overnight at 37 °C. The overnight culture was then transferred to 1 l of LB-Amp and grown at 37 °C to an OD_{600} of 0.6–0.8. Expression of LeuRS was induced with 1 mM IPTG for 3 h at 37 °C. Harvested cells were lysed by sonication and each LeuRS, which contained an N-terminal six-histidine tag, was purified by affinity purification using HIS-Select HF nickel affinity resin as described before [25]. The purified proteins were concentrated via Pierce concentrators 50K MWCO (molecular weight cut-off) (Thermo Scientific, Rockford, IL) and quantitated using the Bio-Rad protein assay according to the commercial protocol.

2.4. Aminoacylation assays

Leucylation reactions contained 60 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 4 μM folded $tRNA^{Leu}$, 21 μM [^{3}H]-L-leucine (150 μCi/ml), and 50 nM enzyme. Initial velocities of leucylation were determined using 10 nM of enzyme. All leucylation reactions were initiated with 4 mM ATP. Aliquots of 10 μl were quenched on TCA-soaked filter pads, washed, and quantitated via scintillation counting as described before [25].

2.5. Mischarged tRNA preparation and enyzmatic deacylation

The [^{3}H]-Ile-$tRNA^{Leu}$ was prepared using an editing defective T252Y LeuRS mutant in an aminoacylation reaction that was incubated at 30 °C for 3 h. Reactions were stopped using 0.18% acetic acid, and extracted using two equal volumes of phenol/chloroform/isoamyl alcohol, pH 4.3 (125:24:1) [26]. A one-half volume of 4.6 M NH_4OAc, pH 5.0, was added followed by an ethanol precipitation. The dried aminoacylated tRNA pellets were resuspended in 10 mM KH_2PO_4, pH 5.0. Hydrolytic editing assays were carried out at room temperature in 60 mM Tris, pH 7.5, 10 mM $MgCl_2$, and 0.8 μM [^{3}H]-Ile-$tRNA^{Leu}$ and