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# Leucyl-tRNA synthetase from the ancestral bacterium *Aquifex aeolicus* contains relics of synthetase evolution

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**The editing reactions catalyzed by aminoacyl-tRNA synthetases are critical for the faithful protein synthesis by correcting misactivated amino acids and misaminoacylated tRNAs. We report that the isolated editing domain of leucyl-tRNA synthetase from the deep-rooted bacterium *Aquifex aeolicus* (αβ-LeuRS) catalyzes the hydrolytic editing of both mischarged $tRNA^{Leu}$ and $minihelix^{Leu}$. Within the domain, we have identified a crucial 20-amino-acid peptide that confers editing capacity when transplanted into the inactive *Escherichia coli* LeuRS editing domain. Likewise, fusion of the β-subunit of αβ-LeuRS to the *E. coli* editing domain activates its editing function. These results suggest that αβ-LeuRS still carries the basic features from a primitive synthetase molecule. It has a remarkable capacity to transfer autonomous active modules, which is consistent with the idea that modern synthetases arose after exchange of small idiosyncratic domains. It also has a unique αβ-heterodimeric structure with separated catalytic and tRNA-binding sites. Such an organization supports the tRNA/synthetase coevolution theory that predicts sequential addition of tRNA and synthetase domains.**

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## Introduction

Aminoacyl-tRNA synthetases (aaRSs) comprise an ancient, diverse enzyme family that catalyzes specific attachment of amino acids to their cognate tRNAs and ensures the accurate translation of the genetic code in the first step of protein synthesis (Carter, 1993; Martinis and Schimmel, 1996). The aminoacylation reaction is accomplished by a two-step process: (a) activation of amino acids with ATP, forming aminoacyl adenylate, and (b) transfer of the aminoacyl residue to the 3′-end of tRNA (Ibba and Söll, 2000). In this two-step reaction, each tRNA synthetase molecule must select and activate its cognate amino acid from the cellular pool of 20 different proteinaceous amino acids. Because of the structural similarity of some amino acids, aaRSs really have difficulties in accurately discriminating cognate substrate from others (Baldwin and Berg, 1966; Loftfield and Vanderjagt, 1972). High fidelity in the amino-acid selection process, which in some cases depends on hydrolytic editing to destroy the misactivated amino acids or mischarged tRNAs, is critical for faithful protein biosynthesis and maintenance of cell survival (Nangle *et al*, 2002).

On the basis of conserved sequence and characteristic structural motifs, aaRSs can be divided into two classes (class I and II) with 10 members in each class (Eriani *et al*, 1990). Several editing systems have been characterized for both classes. For example, class I isoleucyl-tRNA synthetase (IleRS) and valyl-tRNA synthetase (ValRS) have been well studied in terms of their biochemistry and crystal structure (Eldred and Schimmel, 1972; Schmidt and Schimmel, 1994; Nureki *et al*, 1998; Silvian *et al*, 1999; Fukai *et al*, 2000; Hendrickson *et al*, 2000; Tardif *et al*, 2001; Hendrickson *et al*, 2002; Fukunaga *et al*, 2004). More recently, class I leucyl-tRNA synthetase (LeuRS) has also been shown to edit the misactivated noncognate amino acids or the mischarged tRNA (Chen *et al*, 2000, 2001; Mursinna *et al*, 2001; Du and Wang, 2003; Lincecum *et al*, 2003; Xu *et al*, 2004b). Editing activities have been characterized in several class II tRNA synthetases, including threonyl-tRNA synthetase (ThrRS) (Dock-Bregeon *et al*, 2000; Sankaranarayanan *et al*, 2000), prolyl-tRNA synthetase (ProRS) (Beuning and Musier-Forsyth, 2000; Wong *et al*, 2002), and alanyl-tRNA synthetase (AlaRS) (Tsui and Fersht, 1981; Beebe *et al*, 2003).

AaRSs are modular proteins exhibiting different functional roles (Schimmel and Ribas de Pouplana, 2001). It has been proposed that the ancestral aaRS only contained the catalytic domain responsible for adenylate formation and binding to the primitive tRNAs, which were acceptor minihelices (Schimmel and Ribas de Pouplana, 1995). Later in evolution, new domains were appended to or inserted into the catalytic core, increasing the efficiency and accuracy of the aminoacylation process (Schimmel and Ribas de Pouplana, 2001). For certain enzymes, an editing domain was recruited and inserted in the catalytic core to eliminate the misacylated products. Presently, the editing function for subclass Ia, comprising IleRS, LeuRS, and ValRS, is the best documented. These enzymes are thought to have evolved from a common ancestor that did not discriminate between these three amino acids (Heck and Hatfield, 1988; Nureki *et al*, 1998; Cusack *et al*, 2000; Fukai *et al*, 2000). These enzymes contain an unusually large editing domain—also termed connective

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peptide or CP1 (Starzyk *et al*, 1987)—inserted into their catalytic sites. The core of the editing domain has a conserved β-barrel fold, although their peripheral structures are quite different. This similarity suggests that the CP1 domain precursor was probably recruited before the evolutionary divergence of the three enzymes. The insertion of the CP1 domain into the Rossmann fold domain shows some variation across species, suggesting more recent evolutionary shuffling events (Cusack *et al*, 2000). Nevertheless, the CP1 domain was conserved, demonstrating its crucial role for the life of the cell. Isolated CP1 domains from IleRS and ValRS can be expressed as active domains able to deacylate Val-tRNA$^{Ile}$ and Thr-tRNA$^{Val}$, respectively (Lin *et al*, 1996). However, the isolated CP1 domain from *Escherichia coli* LeuRS cannot hydrolyze incorrectly acylated tRNA$^{Leu}$ (Chen *et al*, 2000), despite the fact that LeuRS, IleRS, and ValRS belong to the same enzyme subclass and contain similar CP1 domains.

LeuRS from the ancient bacterium *Aquifex aeolicus* is the only known heterodimeric synthetase, comprised of a 634-amino-acid α-subunit and a 289-amino-acid β-subunit (thus denoted αβ-LeuRS) (Xu *et al*, 2002). The β-subunit of αβ-LeuRS can be expressed as an autonomous thermostable module that binds tRNA$^{Leu}$. The β-subunit also stabilizes the α-subunit when both subunits are coexpressed to form the heterodimeric αβ-LeuRS (Xu *et al*, 2002). The intact αβ-LeuRS can charge both tRNA$^{Leu}$ and minihelices$^{Leu}$ (Xu *et al*, 2004a). Except for the KMSKS sequence, the α-subunit contains the catalytic site for adenylate synthesis and the CP1 domain for editing.

In this study, we examined the editing function of CP1 domains from different LeuRSs. We cloned and expressed the gene fragments encoding the CP1 domains from *A. aeolicus* (hyperthermophilic bacteria), *E. coli* (Gram-negative bacteria), *Thermus thermophilus* (thermophilic bacteria), and *Bacillus subtilis* (Gram-positive bacteria). Rationally chosen mutants of *A. aeolicus* and *E. coli* LeuRSs and their CP1 domains were also generated and tested for their editing functions *in vitro*.

Here, we report that the isolated CP1 domain of *A. aeolicus* αβ-LeuRS is the only molecule able to deacylate the mischarged Ile-tRNA$^{Leu}$. The other bacterial CP1 domains were able to deacylate mischarged Ile-minihelix$^{Leu}$, suggesting that the editing domain of this subclass of aaRSs may have been recruited very early during evolution and then become more and more dependent on the whole enzyme structure. We also identified a 20-amino-acid specific peptide (named the 20-aa motif) in the CP1 domain of *A. aeolicus* αβ-LeuRS, which is crucial for the transfer of the editing capacity to a heterologous CP1 domain. Another transfer of editing capacity was obtained after fusion of an inactive editing domain to the β-subunit of *A. aeolicus* αβ-LeuRS. These results provide new evidence for the evolution of class Ia aaRSs and coevolution with tRNA molecules.

## Results

### *Design of separated CP1 domain and its mutants*

In the X-ray crystal structure of *T. thermophilus* LeuRS (Cusack *et al*, 2000), the CP1 insertion was shown to fold into a discrete domain that is linked to the Rossmann fold-based catalytic core via two β-strand linkers. The linkers are flanked by two conserved $Zn^{2+}$-binding motifs in the class I tRNA synthetase. The putative editing domain was defined as amino-acid residues 224–417, which is a large insertion in the catalytic domain subsequent to the ZN-1 module. The *T. thermophilus* LeuRS shares 49.7, 44.8, and 43.5% identity to the LeuRS enzymes of *A. aeolicus*, *E. coli*, and *B. subtilis*, respectively. All LeuRSs contain a $Zn^{2+}$-binding motif flanking the C-terminus of the CP1 domain. Based on sequence alignments, on *T. thermophilus* LeuRS 3D structure, and on the previous definition of the CP1 domain (Starzyk *et al*, 1987; Burbaum and Schimmel, 1991; Shiba and Schimmel, 1992), we chose and cloned gene fragments containing the CP1 domains of LeuRS from *A. aeolicus* ($A_{125}$–$T_{456}$, *Aa*-CP1), *E. coli* ($A_{126}$–$A_{430}$, *Ec*-CP1), *T. thermophilus* ($T_{126}$–$T_{432}$, *Tt*-CP1), and *B. subtilis* ($N_{125}$–$E_{425}$, *Bs*-CP1) (Figure 1). The four CP1 domains contain the globular editing domain as seen in the 3D structure (Cusack *et al*, 2000) and peptide extensions on both amino- and carboxy-terminal ends as found in the original description of the CP1 domain (Starzyk *et al*, 1987).

In addition, we identified within the *A. aeolicus* CP1 domain, a peptide insertion of 20 amino acids (residues $E_{242}$–$R_{261}$, named the 20-aa motif) that was exclusively found in *A. aeolicus* αβ-LeuRS (Figure 1). Extensive sequence comparisons performed on more than 200 sequences of LeuRS showed that this peptide was only present in the *A. aeolicus* αβ-LeuRS (data not shown). In order to assay its role in editing, we deleted the 20-aa motif from both the *Aa*-CP1 and the intact αβ-LeuRS. These two mutants were named *Aa*-CP1-Δ20 and αβ-LeuRS-Δ20, respectively (Figure 2). We also added the 20-aa motif to the corresponding position in *Ec*-CP1 (between $N_{241}$ and $T_{242}$) to produce another variant named *Ec*-CP1-∇20. Finally, we constructed two chimeric molecules formed by the *Ec*-CP1 fused to the β-subunit of *A. aeolicus* αβ-LeuRS (*Ec*-CP1-β) or to the equivalent polypeptide of the *E. coli* LeuRS (*Ec*-CP1-β′, from $Y_{575}$ to $G_{860}$) (Figure 2) (Zhao *et al*, 2003). The β-subunit is known to contain at least two tRNA$^{Leu}$-binding sites (Xu *et al*, 2002; Zheng *et al*, 2004). Measurements of circular dichroism spectroscopy showed that all these isolated and purified CP1 domains and corresponding mutants were stably folded proteins with α-helices and β-sheet structures (data not shown).

### *Only the A. aeolicus CP1 domain hydrolyzes mischarged Ile-tRNA$^{Leu}$*

After all the CP1 domains were purified, we isolated three mischarged tRNAs: *A. aeolicus* [$^3$H]Ile-tRNA$^{Leu}_{GAG}$, *E. coli* [$^3$H]Ile-tRNA$^{Leu}_{CAG1}$, and *E. coli* [$^3$H]Ile-tRNA$^{Leu}_{CAG2}$. The hydrolytic editing of Ile-tRNA$^{Leu}$ by αβ-LeuRS and all isolated CP1 domains was determined under the following experimental conditions. The αβ-LeuRS (50 nM) was found to deacylate [$^3$H]Ile-tRNA$^{Leu}$ rapidly (Figure 3A). Among the purified CP1 domains, only *Aa*-CP1 (5 μM) was able to deacylate the mischarged *A. aeolicus* tRNA$^{Leu}$ (Figure 3A) and *E. coli* tRNA isoacceptors (Figure 3B), however with a 330-fold decrease of the editing catalytic efficiency as compared with that of αβ-LeuRS (Table I). This loss of editing activity was attributed to both $K_m^{app}$ and $k_{cat}^{app}$ defects, suggesting an effect on the productive tRNA binding. The editing rate catalyzed by the *Aa*-CP1 domain was lower for tRNA$^{Leu}_2$ than that for tRNA$^{Leu}_1$, suggesting that the wobble base pair found at the first position of the acceptor stem of this tRNA might be deleterious for the reaction. Although the *Aa*-CP1 domain shares high homology with other purified bacterial CP1

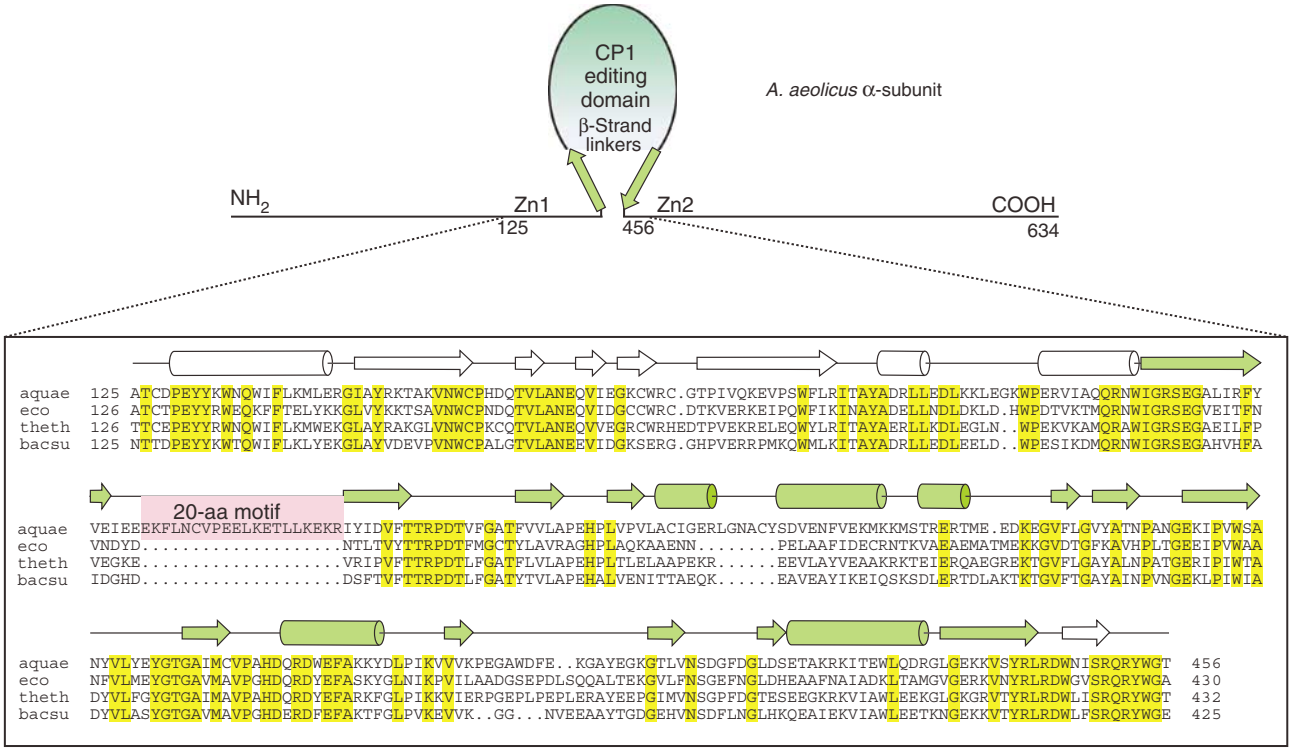


**Figure 1** Schema of *A. aeolicus* LeuRS α-subunit and primary sequence alignment of the studied CP1 domains. The top is the schematic primary sequence of the α-subunit of *A. aeolicus* αβ-LeuRS. Secondary structure elements of the *T. thermophilus* LeuRS are reported on the top line of the alignment (Cusack *et al*, 2000). Those in green correspond to the globular editing domain. Strictly conserved residues among the four bacterial species are colored in yellow. The 20-aa peptide specific to the *Aquifex* enzyme is highlighted in purple. Organisms' abbreviations are as follows: aquae, *A. aeolicus*; eco, *E. coli*; theth, *T. thermophilus*; bacsu, *B. subtilis*).

domains, the deacylation of Ile-tRNA$^{Leu}$ was never observed even when the concentrations of these separated CP1 domains were increased to 15 μM (data not shown). We cannot exclude that this defect in editing activity might be due to unproductive cross-species interactions with the noncognate *A. aeolicus* tRNA$^{Ile}$. Another possibility might be that the peptide extensions located on both sides of the globular editing domains (Figure 1) influence the structure or the activity of tRNA editing. However, the presence of similar extensions in IleRS- and ValRS-CP1 domains does not impair the editing activity (Lin *et al*, 1996) and moreover, in the case of LeuRS, the circular dichroism spectroscopy analyses have shown that the isolated CP1 domains are stably folded (data not shown).

### *The β-subunit of the A. aeolicus LeuRS rescues the E. coli CP1 deficiency*

In the following experiments, we showed that the editing activity of the *E. coli* CP1 domain could be conferred by fusion with RNA-binding domains. The *Ec*-CP1-β chimera is a fusion between the *E. coli* CP1 domain and the β-subunit comprising the tRNA-binding sites of αβ-LeuRS. Although *Ec*-CP1 did not deacylate the mischarged *A. aeolicus* and *E. coli* Ile-tRNAs, *Ec*-CP1-β (5 μM) deacylated Ile-tRNA$^{Leu}$ efficiently (Figure 4A, Table II): the catalytic efficiency of *Ec*-CP1-β in editing *A. aeolicus* Ile-tRNA$^{Leu}$ was 10 times higher than that of *Aa*-CP1. On the other hand, hydrolytic editing for *E. coli* Ile-tRNA$^{Leu}$ was 20 times lower than for *A. aeolicus* Ile-tRNA$^{Leu}$, suggesting improper binding of the *E. coli* tRNA on the *A. aeolicus* β-subunit. We have previously shown that the β-subunit can bind both *A. aeolicus* and *E. coli* tRNA$^{Leu}$s *in vitro* (Xu *et al*, 2002), but only *A. aeolicus* tRNA$^{Leu}$ *in vivo* (Zheng *et al*, 2004). Thus, it is likely that the *Ec*-CP1-β mutant prefers to hydrolyze the mischarged *A. aeolicus* tRNA$^{Leu}$ due to the intrinsic preference of the β-subunit for its own tRNA.

A second fusion between the *E. coli* CP1 and the *E. coli* β-like polypeptide was also constructed (*Ec*-CP1-β′), but the editing activity of *Ec*-CP1-β′ for the *A. aeolicus* and *E. coli* mischarged tRNA$^{Leu}$ reached only 1/31 and 1/37, respectively, of the editing activity of *Ec*-CP1-β for *A. aeolicus* Ile-tRNA$^{Leu}$, and no preference was observed for the *E. coli* tRNA (Figure 4B, Table II). These data suggest that *Ec*-CP1 carries a latent editing activity but needs some 'codomain' to perhaps stabilize the binding of the tRNA substrate. The β-subunit that binds the tRNA in *trans* in the αβ-LeuRS may play the role of this codomain acting in *cis* in the fusion protein.

### *The 20-aa motif specific to the A. aeolicus CP1 domain is crucial for the editing of Ile-tRNA$^{Leu}$*

The 20-aa peptide insertion extending from residues 242 to 261 is specific for the CP1 domain of *A. aeolicus* αβ-LeuRS (Figure 1). To investigate the effect of this insertion on the hydrolytic editing of both *Aa*-CP1 and αβ-LeuRS, two deletion mutants named *Aa*-CP1-Δ20 and αβ-LeuRS-Δ20 were constructed and purified. *Aa*-CP1-Δ20 dramatically decreased the deacylation of the mischarged *A. aeolicus* Ile-tRNA$^{Leu}$ when compared with *Aa*-CP1 (Figure 5A). The $k_{cat}^{app}$ and $k_{cat}^{app}/K_m^{app}$ of *Aa*-CP1-Δ20 in the reaction were reduced to 20 and 14.3% of *Aa*-CP1, respectively (Table I). However, in the αβ-LeuRS-Δ20 mutant, this deletion had no effect on editing ability (Table I).

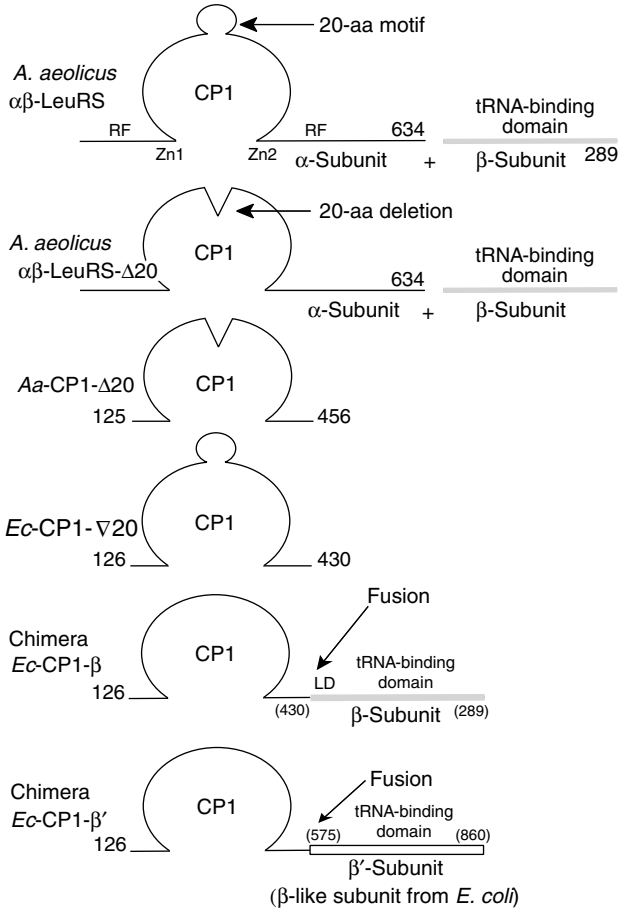


**Figure 2** Schematic representation of the different constructed mutants. α-Subunit contains the editing domain (CP1), Rossmann fold (RF), Zinc-binding domains (Zn1 and Zn2), leucyl-specific domain (LD), and β-subunit is the tRNA-binding domain of *A. aeolicus* αβ-LeuRS. αβ-LeuRS-Δ20 is an αβ-LeuRS with the deletion of the 20-aa motif. *Aa*-CP1-Δ20 represents the deletion mutant of the 20-aa motif in *Aa*-CP1. *Ec*-CP1-∇20 contains insertion of the 20-aa motif, whereas the chimeric *Ec*-CP1-β and *Ec*-CP1-β′ are *E. coli* CP1 domains fused to the β-subunit of the *A. aeolicus* LeuRS and β-like subunit (β′) of *E. coli* LeuRS (Zhao *et al*, 2003), respectively.

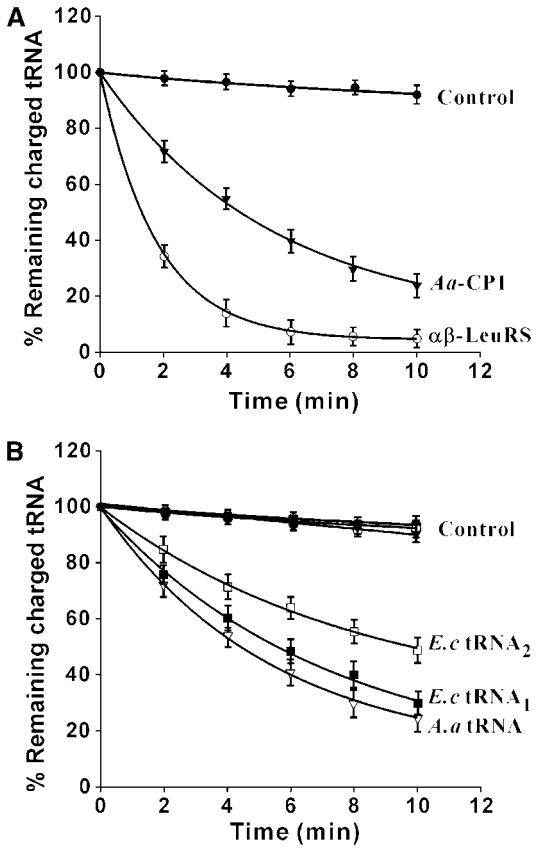


**Figure 3** Deacylation of mischarged [$^3$H]Ile-tRNA$^{Leu}$ by *Aa*-CP1 and αβ-LeuRS. Reactions were carried out at 37°C and no enzymes or CP1 domains were added to the control reactions. (**A**) Deacylation of mischarged *A. aeolicus* tRNA$^{Leu}$ by *Aa*-CP1 (5 μM) and αβ-LeuRS (50 nM). (**B**) Deacylation of different mischarged tRNAs (*E.c* tRNA$_{1,2}$, *E. coli* tRNA$_1^{Leu}$ or tRNA$_2^{Leu}$; *A.a* tRNA, *A. aeolicus* tRNA$^{Leu}$) by *Aa*-CP1 (5 μM). Values were obtained from three independent determinations.

The results showed that this 20-aa motif is only important for the hydrolytic editing function of the isolated *Aa*-CP1 domain.

Conversely, we asked whether the addition of this peptide to the *Ec*-CP1 domain would confer editing ability. We constructed the insertion mutant *Ec*-CP1-∇20 in which the 20-aa peptide was inserted between residues $N_{242}$ and $T_{243}$ and examined for editing activity. Remarkably, we found that *Ec*-CP1-∇20 could edit the mischarged Ile-tRNA$^{Leu}$ efficiently (Figure 5B), indicating that the 20-aa motif is also essential for the editing activity of heterologous CP1 domains. Comparison of the editing parameters of *Ec*-CP1-∇20 mutant and the isolated *Aa*-CP1 revealed that transplantation of the 20-aa motif confers to the *E. coli* CP1 an editing activity that is very close to that of *Aa*-CP1 (Tables I and III). Nevertheless, a slight preference for the editing of *A. aeolicus* Ile-tRNA$^{Leu}$ was observed when comparing with the editing for the two *E. coli* tRNAs (Table III). Therefore, it appears that the 20-aa motif from the *A. aeolicus* CP1 domain preferentially targets mischarged tRNA originating from *A. aeolicus*.

### *Both A. aeolicus and E. coli CP1 domains can edit mischarged Ile-minihelix$^{Leu}$*

The two different domains of tRNA, the acceptor-TΨC stem–loop domain and the D-anticodon stem–biloop domain, interact with the catalytic site and the anticodon binding module of aaRS, respectively. According to the hypothesis that these two tRNA domains may have arisen independently (Buechter and Schimmel, 1993), acceptor minihelices are chargeable by aaRSs (Francklyn and Schimmel, 1989) and mischargeable by heterologous aaRSs (Nordin and Schimmel, 1999). In a recent report, we showed that the *Aa*-Δ1:C72 minihelix$^{Leu}$ (Figure 6A) is efficiently aminoacylated by αβ-LeuRS (Xu *et al*, 2004a). Because the minihelices$^{Ile}$ are recognized by IleRS at the level of the identity element A73 (Nordin and Schimmel, 1999), we made the assumption that IleRS can also transfer Ile onto the *Aa*-Δ1:C72 minihelix$^{Leu}$ (with A73) and therefore produce Ile-Δ1:C72 minihelix$^{Leu}$. As expected, the Ile-Δ1:C72 minihelix$^{Leu}$ was formed by IleRS and this mischarged substrate was used in the editing assay with various CP1 domains. For simplification, the *Aa*-Δ1:C72 minihelix$^{Leu}$ was named minihelix$^{Leu}$, since only one minihelix$^{Leu}$ was used in this work. Editing activity was

**Table I** Apparent kinetic parameters for hydrolytic editing of mischarged *A. aeolicus* [$^3$H]Ile-tRNA$^{Leu}_{GAG}$

| Proteins | $k_{cat}^{app}$ ($s^{-1}$) | $K_m^{app}$ (μM) | $k_{cat}^{app}/K_m^{app}$ ($\mu M^{-1}\,s^{-1}$) | $k_{cat}^{app}/K_m^{app}$ (relative) | Loss of efficiency (-fold) |
|---|---|---|---|---|---|
| LeuRS | | | | | |
| αβ-LeuRS | 1.30±0.12 | 1.70±0.25 | 0.76 | 1 | 1 |
| αβ-LeuRS-Δ20 | 1.20±0.10 | 1.50±0.22 | 0.80 | 1.05 | 0.95 |
| CP1 | | | | | |
| *Aa*-CP1 | $5.0\pm0.7\times10^{-2}$ | 21±2.8 | $2.38\times10^{-3}$ | $3.0\times10^{-3}$ | 330 |
| *Aa*-CP1-Δ20 | $1.0\pm0.2\times10^{-2}$ | 30±3.2 | $0.33\times10^{-3}$ | $4.3\times10^{-4}$ | 2303 |

Catalytic efficiency values from five independent determinations were within ±10%. $k_{cat}^{app}$ and $K_m^{app}$ are apparent $k_{cat}$ and $K_m$, respectively.

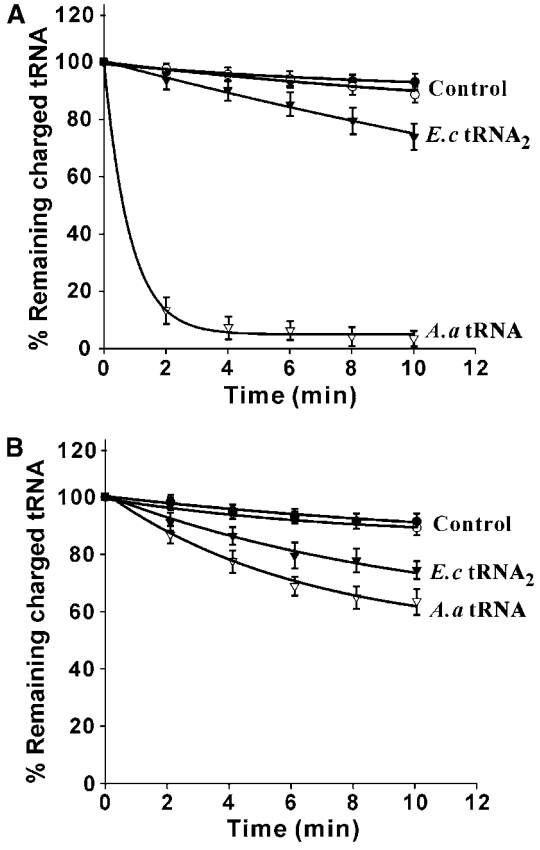


**Figure 4** Analysis of the editing activities of the chimeric proteins *Ec*-CP1-β and *Ec*-CP1-β′. Deacylation reactions were carried out at 37°C with [$^3$H]Ile-tRNAs as substrates. (**A**) *Ec*-CP1-β (5 μM) was used to deacylate mischarged *E. coli* tRNA$_2^{Leu}$ and *A. aeolicus* tRNA$^{Leu}$. (**B**) *Ec*-CP1-β′ (5 μM) was used to deacylate mischarged *E. coli* tRNA$_2^{Leu}$ and *A. aeolicus* tRNA$^{Leu}$. Values were obtained from three independent determinations. *E. coli* tRNA$_1^{Leu}$ was deacylated at the same rate as *E. coli* tRNA$_2^{Leu}$.

measured using 5 μM of the different CP1 domains. We found that all bacterial CP1 domains were able to hydrolyze the Ile-minihelix$^{Leu}$, including *Aa*-CP1, *Ec*-CP1, *Bs*-CP1, and *Tt*-CP1 (Figure 6B).

In contrast to the experiments performed with mischarged tRNAs, it now appears that the mischarged minihelix is equally deacylated by the intact αβ-LeuRS and the isolated *Aa*-CP1 domain with the same concentration of 5 μM (Figure 6B). In fact, the editing rate of the whole enzyme was decreased, while the *Aa*-CP1 editing rate specifically increased toward the mischarged minihelix.

### *The editing activity for minihelices can be improved by appending or inserting extra peptides on the CP1 domain*

In this part of the study, we showed that the 20-aa motif is essential for the editing of mischarged minihelices catalyzed by *Aa*-CP1 and *Ec*-CP1.

Using a 20-aa deletion mutant of *Aa*-CP1 (*Aa*-CP1-Δ20), we showed that minihelix$^{Leu}$ editing was decreased (Figure 7A). Under the same conditions, *Ec*-CP1 can deacylate the mischarged minihelix$^{Leu}$, although at a lower rate than the insertion *Ec*-CP1-∇20 mutant (Figure 7B). Therefore, the 20-aa motif favors the editing of mischarged minihelices$^{Leu}$.

We also examined the consequence of appending the β- and β′-subunit to the *Ec*-CP1 domain (*Ec*-CP1-β and *Ec*-CP1-β′ mutants) on the editing of the mischarged minihelix. However, the editing properties of the mutants did not differ significantly from that of *Ec*-CP1, suggesting that the β-subunit does not participate in productive minihelix binding (data not shown).

Altogether, the results show the importance of accessory peptides for the editing activity of the isolated CP1 domain. The 20-aa motif can confer editing capacity for the mischarged tRNA and minihelix when transplanted in inactive CP1 domains. Likewise, the β-subunit fused to an inactive CP1 domain can confer tRNA editing activity.

## Discussion

### *Functional modules of A. aeolicus LeuRS suggest a pathway for assembly of aaRSs*

AaRSs comprise a family of modular enzymes composed of domains that have distinct roles in the aminoacylation reactions (Delarue and Moras, 1993). The two classes of aaRSs known to date display two types of catalytic cores that are thought to be the original and historical part of the aaRSs that contained the elementary structural components needed for adenylate formation. These cores are aided by a variety of additional domains that contact the tRNA and provide specificity and efficiency in translation. These RNA-binding elements are imagined to have been added to allow early RNA substrates the ability to dock in close vicinity to the activated amino acid and promote its transfer to the tRNAs. In parallel, some aaRSs have evolved editing mechanisms to prevent errors in protein synthesis and to favor faithful decoding of genetic information (Nangle *et al*, 2002). There are two possible ways with which editing can be achieved. The first is pretransfer editing: the misactivated amino acid is directly hydrolyzed by aaRS (Fersht, 1977). The second possibility is post-transfer editing, occurring after the transfer of the misactivated amino acid on the 3′ end of the tRNA and consisting

 

**Table II** Apparent kinetic parameters of *Ec*-CP1-β and *Ec*-CP1-β′ in editing mischarged [$^3$H]Ile-tRNA$^{Leu}_{GAG}$

| Protein | tRNA$^{Leu}$ origin | $k^{app}_{cat}$ ($s^{-1}$) | $K^{app}_m$ (μM) | $k^{app}_{cat}/K^{app}_m$ ($\mu M^{-1} s^{-1}$) | $k^{app}_{cat}/K^{app}_m$ (relative) | Loss of efficiency (-fold) |
|---|---|---|---|---|---|---|
| *Ec*-CP1-β | *A. aeolicus* | $0.12 \pm 0.02$ | $5.60 \pm 0.5$ | $21.4 \times 10^{-3}$ | 1 | 1 |
| | *E. coli* | $1.7 \pm 0.2 \times 10^{-2}$ | $16 \pm 2.8$ | $1.06 \times 10^{-3}$ | 0.05 | 20 |
| *Ec*-CP1-β′ | *A. aeolicus* | $2.1 \pm 0.3 \times 10^{-2}$ | $31 \pm 3.8$ | $0.68 \times 10^{-3}$ | 0.032 | 31 |
| | *E. coli* | $1.6 \pm 0.2 \times 10^{-2}$ | $28 \pm 4.1$ | $0.57 \times 10^{-3}$ | 0.027 | 37 |

Relative $k^{app}_{cat}/K^{app}_m$ and loss of efficiency values were expressed according to the most efficient catalyst (*Ec*-CP1-β with *A. aeolicus* tRNA$^{Leu}$). All values are the average of three experiments. *E. coli* tRNA$^{Leu}_2$ was the isoacceptor used in the assays. $k^{app}_{cat}$ and $K^{app}_m$ are apparent $k_{cat}$ and $K_m$, respectively.

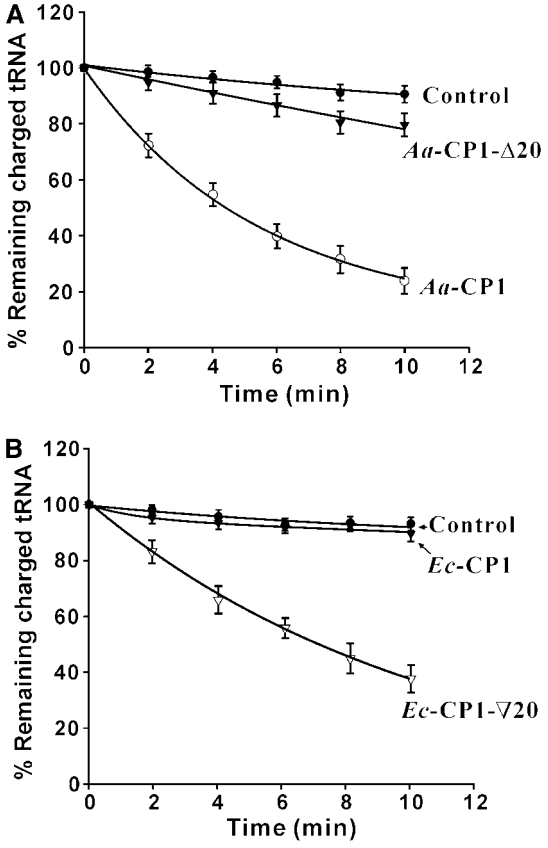


**Figure 5** Editing activities of the isolated CP1 domains and mutants. Deacylation of [$^3$H]Ile-tRNA$^{Leu}$ was measured at 37°C with a concentration of 5 μM of isolated CP1 domains. (**A**) Deletion of the 20-aa motif in mutant *Aa*-CP1-Δ20 reduces the editing capacity of mischarged *A. aeolicus* tRNA$^{Leu}$ compared to the native *Aa*-CP1 domain. (**B**) The insertion of the 20-aa motif confers editing capacity to mutant *Ec*-CP1-∇20 compared to *Ec*-CP1.

of hydrolysis of the misaminoacyl bond (Eldred and Schimmel, 1972). For many aaRSs, the proofreading properties result from a combination of the two routes.

In the present study, we examined the post-transfer editing of four distinct bacterial LeuRSs. A previous attempt to isolate an active CP1 domain from LeuRS was unsuccessful (Chen *et al*, 2000) despite the fact that the isolated CP1 domains from the two related synthetases IleRS and ValRS were active (Lin *et al*, 1996). We found that only the CP1 domain of the heterodimeric αβ-LeuRS from the ancestral bacterium *A. aeolicus* is active as an isolated domain. This finding appears to be rather exceptional considering that the three other bacterial CP1 domains were inactive in proofreading. Phylogenetic analyses place the genus *Aquifex* as the deepest branch of the bacterial phylogenetic tree (Deckert *et al*, 1998), having retained many peculiar proteins and mechanisms thought to be relics from primitive organisms. Here, we propose that this exceptional result, connected to the LeuRS anomaly in *A. aeolicus*—the heterodimeric αβ-structure of LeuRS—might support theories on the design and evolution of tRNA synthetases and on the mechanisms by which they recognize and transfer amino acids onto tRNA$^{Leu}$. The assembly of the two subunits of *A. aeolicus* LeuRS might represent a general pathway for the acquisition of RNA-binding domains by aaRSs during evolution (Zhao *et al*, 2003). Indeed, if modern tRNAs form an L-shaped structure consisting of two domains, which are the acceptor-TΨC stem–loop domain (acceptor minihelix) and the D-anticodon stem–biloop domain (SBL), it is possible that these two domains arose independently with the acceptor minihelix as the earliest known substrate for aminoacylation (Schimmel, 1993). According to this hypothesis, ancient aaRSs might have been mini-aaRSs, similar to the α-subunit of the *A. aeolicus* LeuRS, containing almost the entire catalytic core. Subsequently, tRNAs and aaRSs were assembled by joining the RNA and peptide domains to primitive minihelices and catalytic cores, respectively (Schimmel and Ribas de Pouplana, 1995). According to this scheme, the presence of a distinct β-subunit in the *A. aeolicus* LeuRS that independently binds tRNA$^{Leu}$ *in vitro* (Xu *et al*, 2002) and *in vivo* (Zheng *et al*, 2004) illustrates the adaptation of enzymes to tRNA evolution.

Other evidence supports the tRNA-synthetase coevolution theory. As a relic of the ancestral mini-aaRSs, more than half of the modern aaRSs have preserved their charging activity for acceptor minihelices or smaller 7-bp RNA fragments called microhelices (Francklyn and Schimmel, 1989; Martinis and Schimmel, 1992). αβ-LeuRS is the only known LeuRS that can leucylate minihelices (Xu *et al*, 2004a) similar to an ancient aaRS. Taken together, these data show that *A. aeolicus* αβ-LeuRS has conserved at least three ancestral features from a primitive synthetase, which are aminoacylation of minihelices, tRNA editing by an isolated CP1 domain, and tRNA binding by an isolated β-subunit. Thus, the example of *A. aeolicus* αβ-LeuRS strongly argues for a model of construction of aaRSs based on the acquisition of independent and specialized modules during evolution.

### *Activation of E. coli editing domain by A. aeolicus peptides might mimic the process of synthetase evolution*

We investigated the hydrolytic properties of several CP1 domains originating from different LeuRSs (see Figure 1);

**Table III** Apparent editing parameters of *Ec*-CP1-∇20 mutant for mischarged [$^3$H]Ile-tRNA$^{Leu}$

| Mischarged tRNA | $k_{cat}^{app}$ ($s^{-1}$) | $K_m^{app}$ (μM) | $k_{cat}^{app}/K_m^{app}$ ($\mu M^{-1} s^{-1}$) | $k_{cat}^{app}/K_m^{app}$ (relative) | Loss of efficiency (-fold) |
|---|---|---|---|---|---|
| *A. aeolicus* tRNA$^{Leu}$ | $3.8 \pm 0.6 \times 10^{-2}$ | $27 \pm 3.8$ | $1.4 \times 10^{-3}$ | 1 | 1 |
| *E. coli* tRNA$_1^{Leu}$ | $2.3 \pm 0.4 \times 10^{-2}$ | $32 \pm 4.2$ | $0.7 \times 10^{-3}$ | 0.50 | 2 |
| *E. coli* tRNA$_2^{Leu}$ | $2.2 \pm 0.3 \times 10^{-2}$ | $35 \pm 4.8$ | $0.6 \times 10^{-3}$ | 0.43 | 2.3 |

Data are the average value from three independent determinations. $k_{cat}^{app}$ and $K_m^{app}$ are apparent $k_{cat}$ and $K_m$, respectively.

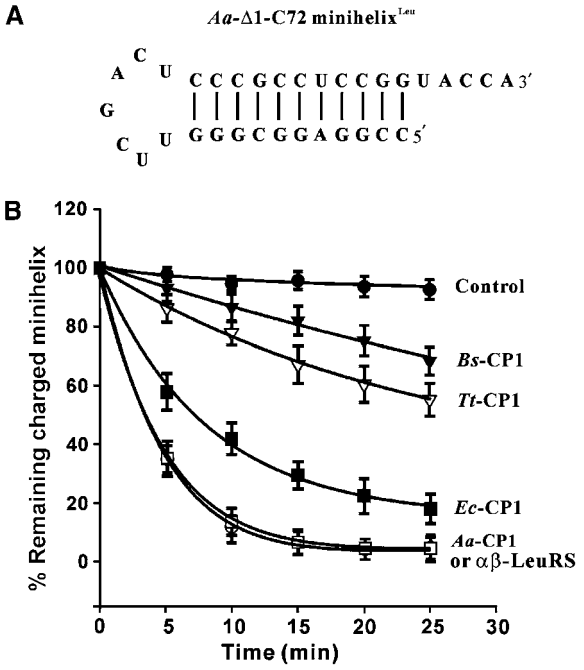


**Figure 6** Deacylation of mischarged [$^3$H]Ile-minihelix$^{Leu}$ by different isolated CP1 domains and αβ-LeuRS. Minihelix$^{Leu}$ used is shown on the top of the figure (**A**) (Xu *et al*, 2004a). Measurements were performed at 25°C with a final concentration of 5 μM of the different proteins (**B**). Mischarged minihelix is resistant to deacylation in the absence of enzyme (control curve). Curves resulted from three independent determinations.

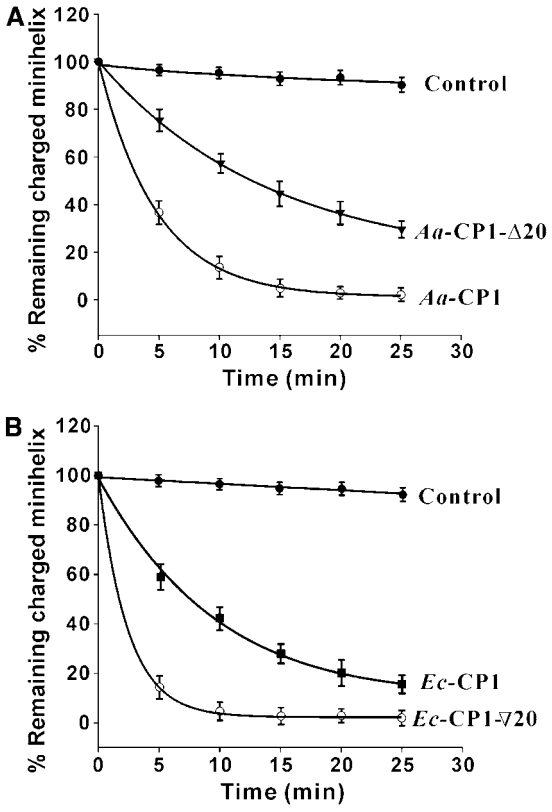


**Figure 7** Deacylation of mischarged [$^3$H]Ile-minihelix$^{Leu}$ by different CP1 mutants. Reactions were carried out at 25°C with 5 μM of isolated CP1 domains or LeuRSs. (**A**) Deletion of the 20-aa motif in *Aa*-CP1 (named *Aa*-CP1-Δ20) reduced hydrolytic editing. (**B**) Insertion of the 20-aa motif in *Ec*-CP1 (named *Ec*-CP1-∇20) improved the hydrolytic editing. Values were obtained from three independent determinations.

however, except for *Aa*-CP1, none were able to carry out the tRNA editing reaction. We identified two *A. aeolicus* peptides in the αβ-LeuRS that activated *Ec*-CP1 when appended or inserted into the CP1 domain.

One element was the β-subunit from the *A. aeolicus* LeuRS that typically binds tRNA in the class Ia synthetases. Structural analyses of the two related IleRS and ValRS have shown that the helix-bundle region contained in this domain binds the anticodon stem–loop of the cognate tRNA (Silvian *et al*, 1999; Fukai *et al*, 2000). In most LeuRSs, the helix-bundle scaffold is not used to recognize specifically the anticodon nucleotides of tRNA$^{Leu}$ (Asahara *et al*, 1993; Soma *et al*, 1999; Tocchini-Valentini *et al*, 2000). However, the existence of unspecific interactions with the sugar–phosphate backbone of the tRNA is not excluded. For instance, in *A. aeolicus* LeuRS, interactions with tRNA$^{Leu}$ have been shown by the functional and genetic studies performed on the β-subunit (Xu *et al*, 2002; Zheng *et al*, 2004). In this study, we show that the β-subunit can confer editing activity to the *Ec*-CP1 domain when fused to it (mutant *Ec*-CP1-β). This suggests that the β-subunit stabilizes the interaction occurring between the isolated CP1 domain and the mischarged tRNA substrate.

The second structural element that we identified in the CP1 domain of *A. aeolicus* αβ-LeuRS was a specific 20-amino-acid peptide. Its insertion into the isolated inactive CP1 domain from *E. coli* (mutant *Ec*-CP1-∇20) confers editing activity. Remarkably, the 20-aa motif is dispensable in intact αβ-LeuRS, suggesting that this cryptic activity is another relic from a time when the CP1 domains were not inserted into the catalytic cores but were present as free domains catalyzing *trans*-acting editing of misacylated tRNAs. Recent studies have shown that autonomous editing domains still exist, but compared to inserted editing domains, they exhibit less tRNA specificity and efficiency (Ahel *et al*, 2003; Wong *et al*, 2003; An and Musier-Forsyth, 2004; Korencic *et al*, 2004). During evolution of LeuRSs, the 20-aa motif presumably disappeared from the other bacterial LeuRSs due to the acquisition of new tRNA-binding domains that helped to stabilize the editing activity. The absence of the 20-aa motif in the *T. thermophilus* LeuRS sequence leads to speculation

 

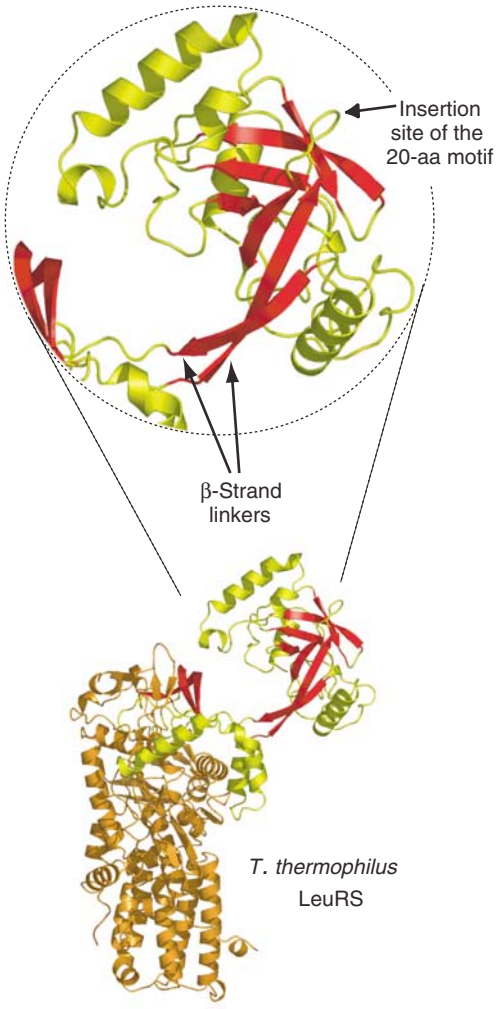


**Figure 8** Overview of the *T. thermophilus* LeuRS and detailed view of its CP1 domain. The lower part of the figure depicts the *T. thermophilus* LeuRS, showing the large size and globular nature of the editing domain (Protein Data Bank (PDB) ID code: 1H3N) (Cusack *et al*, 2000). The studied CP1 domain is colored yellow (α-helices and loops) and red (β-strands). The other domains of the molecule are colored orange. The upper part of the figure is a detailed view of the editing domain. The two β-strand linkers that link the editing domain to the catalytic site are indicated, as well as is the insertion point of the crucial '20-aa motif' specific for *A. aeolicus* LeuRS.

about its 3D location and folding. The *T. thermophilus* structure shows that the motif is inserted in a turn that connects the first long flexible β-strand to the second antiparallel β-strand (Figure 8). This couple of strands forms a partially solvent-exposed β-linker that links the CP1 domain to the active site domain. It has been proposed that β-strand flexibility might be implicated in the editing reaction process, as suggested by the significant rotation of the CP1 domain that is observed in the presence of tRNA (Nureki *et al*, 1998; Silvian *et al*, 1999; Cusack *et al*, 2000; Fukunaga and Yokoyama, 2005). Here, we observed that the insertion of the 20-aa motif in the β-ribbon turn of the *E. coli* enzyme activates its editing capacity. Two-dimensional models predict that the 20-aa motif might be folded as a small β-strand followed by a long amphiphilic α-helix of about 12 residues (data not shown). The 20-aa motif could stabilize or strengthen the CP1 structure. It might also extend the β-strand linker that connects the editing site to the catalytic site and modify the flexibility of the domain. The effect might be more productive binding of the tRNA in the editing site. However, the presence of editing activity in all the tested bacterial CP1 domains toward the minihelix$^{Leu}$ suggests that these CP1 structures are already active, at least for the binding and editing of minihelices. Thus, the 20-aa motif should presumably influence, either directly or indirectly, the binding of the intact tRNA molecule.

The experiments reported here support the idea that peptide or module fusion with a domain catalyzing the hydrolytic editing reaction of a primitive minihelix could result in the acquisition of tRNA editing function. Thus, autonomous tRNA editing domains could have been assembled in early evolution of development of the aminoacylation systems and genetic code, when aaRSs were not perfectly specific. These independent domains could then have been combined with primitive aaRSs in order to increase specificity and efficiency of both synthetic and editing functions. Other groups have proposed comparable domain fusions to create synthetic catalytic sites in primitive aaRSs. It has been shown that large nonspecific RNA-binding domains of 175 and 228 aa (from Arc1p and GluRS, respectively), fused to the catalytic domain of AlaRS for the synthesis of alanyl-adenylate, can produce a chimera able to catalyze aminoacylation of the microhelix$^{Ala}$ (Chihade and Schimmel, 1999). In another example, the fusion of the catalytic domain to an 'artificial' peptide sequence (28 aa) specific of tRNA$^{Ala}$ binding confers aminoacylation activity and specificity for hairpin microhelices exhibiting the G3:U70 base pair (Frugier *et al*, 2003). Taken together, these studies suggested that starting from a simple catalytic site, barriers to add new RNA-binding sites or new catalytic function are relatively low. Synthetic or editing catalytic sites can be created and improved by sequential addition of new functional units. These findings also support the idea that conformational flexibility is inherent to the added modules, and suggest the possibility that such flexibility can be incorporated into structures and play a role in signal transduction. Studies performed with modern aaRSs have confirmed the principle of long-range communication between the tRNA-binding domain and the catalytic site (Alexander and Schimmel, 1999; Eriani and Gangloff, 1999; Steer and Schimmel, 1999) and it has been proposed that interdomain signaling is an early event that has played a significant role in the historical assembly of the tRNA/synthetase complexes (Steer and Schimmel, 1999).

## Materials and methods

### *Materials*

L-Isoleucine, 5′-GMP, ATP, GTP, CTP, UTP, tetrasodium pyrophosphate, inorganic pyrophosphatase, and DTT were purchased from Sigma (USA). L-[$^3$H]isoleucine (1 mCi/ml) and L-[$^3$H]leucine were obtained from Amersham Life Sciences (England). Kinase, ligase, RNasin (ribonuclease inhibitor), isopropyl β-D-thiogalactoside (IPTG), and all restriction endonucleases were obtained from Sangon Company, Shanghai Branch, Canada. T7 RNA polymerase was purified as described (Li *et al*, 1999). Plasmids pET28a and pET30a were purchased from Novagen (Biosciences Inc.). The bacterial BL21-Codon Plus (DE3)-RIL strain was purchased from Stratagene (USA). Nickel-nitrilotriacetic (Ni-NTA) Superflow resin was obtained from Qiagen Inc. (Germany). GF/C and DE-81 filters were obtained from the Whatman Company (UK). Genomic DNA

from *T. thermophilus* was a gift from Dr M Sprinzl (Universitate Bayreuth, Germany).

#### *Cloning of CP1 domain genes*

The DNA fragments encoding CP1 domains were amplified by PCR using either plasmid or genomic DNA as a template. Products were cloned in either pET28a or pET30a. The DNA fragments encoding the fusion protein of *E. coli* CP1 domain (*Ec*-CP1) with *A. aeolicus* β-subunit and *E. coli* β-like polypeptide were inserted into pET30a by a two-step procedure. The mutant CP1 domain deletion in *A. aeolicus* αβ-LeuRS was constructed as described previously (Zhao *et al*, 2003).

#### *Protein preparation*

All the cloned CP1 domain genes were overexpressed in the *E. coli* BL21-Codon Plus (DE3)-RIL strain. Cells from overnight cultures were diluted 100-fold, grown at 37°C to mid-log phase, and induced with 80 μM IPTG for 5 h at 25°C. Cells were harvested by centrifugation, lysed by sonication, and purified by Ni-NTA chromatography as described previously (Chen *et al*, 1999).

#### *RNA substrate preparation*

The *in vitro* transcriptions of *A. aeolicus* $tRNA^{Leu}$, *E. coli* $tRNA_1^{Leu}$, $tRNA_2^{Leu}$, and $minihelix^{Leu}$ were performed as described previously (Xu *et al*, 2004a). The mischarged [$^3$H]Ile-$tRNA^{Leu}$ was prepared using the *E. coli* LeuRS mutant LeuRS-$T_{252}$E (Xu *et al*, 2004b). Leu-$tRNA^{Leu}$ was prepared using wild-type *E. coli* LeuRS (Xu *et al*, 2004b). For the minihelix, we used the most active *Aa*-Δ1-C72 $minihelix^{Leu}$ as a substrate (Xu *et al*, 2004a). The mischarged [$^3$H]Ile-$minihelix^{Leu}$ was prepared by the isoleucylation of $minihelix^{Leu}$ with *E. coli* IleRS as described previously (Nordin and Schimmel, 1999).

#### *Hydrolytic editing assays*

The hydrolytic editing activities of different CP1 domains toward mischarged [$^3$H]Ile-$tRNA^{Leu}$ were measured in reactions containing 100 mM Tris–HCl, pH 7.5, 30 mM KCl, 12 mM $MgCl_2$, 0.5 mM DTT, and 1 μM [$^3$H]Ile-$tRNA^{Leu}$ (~270 μCi/μmol) at 37°C. The reaction was initiated by adding 5 μM of CP1 domain or 50 nM LeuRS. At various time intervals, aliquots were quenched and precipitated with 5% trichloroacetic acid (TCA) as described previously (Xu *et al*, 2002). The kinetic parameters for the deacylation reactions of the mischarged Ile-$tRNA^{Leu}$ were determined using a range of 1–80 μM [$^3$H]Ile-$tRNA^{Leu}$ and 50 nM enzyme or 0.5 μM CP1 domain. For mischarged Ile-$minihelix^{Leu}$, reactions containing 1 μM [$^3$H]Ile-$minihelix^{Leu}$ (~20 mCi/μmol) were initiated by the addition of 5 μM CP1 domain or enzyme at 25°C. At various time intervals, aliquots were quenched by spotting on DE81 Whatman filter for 3.5 min before washing with cold 5% TCA (Xu *et al*, 2004a).

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