A dispensable peptide from *Acidithiobacillus ferrooxidans* tryptophanyl-tRNA synthetase affects tRNA binding

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Abstract **The activation domain of class I aminoacyl-tRNA synthetases, which contains the Rossmann fold and the signature sequences HIGH and KMSKS, is generally split into two halves by the connective peptides (CP1, CP2) whose amino acid sequences are idiosyncratic. CP1 has been shown to participate in the binding of tRNA as well as the editing of the reaction intermediate aminoacyl-AMP or the aminoacyl-tRNA. No function has been assigned to CP2. The amino acid sequence of *Acidithiobacillus ferrooxidans* TrpRS was predicted from the genome sequence. Protein sequence alignments revealed that *A. ferrooxidans* TrpRS contains a 70 amino acids long CP2 that is not found in any other bacterial TrpRS. However, a CP2 in the same relative position was found in the predicted sequence of several archaeal TrpRSs. *A. ferrooxidans* TrpRS is functional in vivo in *Escherichia coli*. A deletion mutant of *A. ferrooxidans trpS* lacking the coding region of CP2 was constructed. The in vivo activity of the mutant TrpRS in *E. coli*, as well as the kinetic parameters of the in vitro activation of tryptophan by ATP, were not altered by the deletion. However, the K_m value for tRNA was seven-fold higher upon deletion, reducing the efficiency of aminoacylation. Structural modeling suggests that CP2 binds to the inner corner of the L shape of tRNA.**

Key words: Aminoacyl-tRNA synthetase; Deletion mutant; Peptide function

1. Introduction

Aminoacyl-tRNA synthetases (AARS) are the key enzymes to ensure the faithful translation of genetic information. With the exception of glutamine and asparagine, most organisms contain at least one copy of the genes encoding the enzymes that aminoacylate each amino acid to the 3′ end of the cognate tRNA. The aminoacylation of tRNA by the synthetases occurs in a two-step reaction, activation of the amino acid with ATP and subsequent transfer of the activated residue to the 3′ end of tRNA. At least two domains can be visualized in all AARS, the activation or catalytic domain and the anticodon binding domain. Based on the common structure of the activation domains, AARS are divided into two classes. In class I, the active site is composed of a parallel β-sheet flanked by α-helices at both ends (Rossmann fold). In class II, the active site is made up of an antiparallel β-sheet [1]. Within the domains, signature sequences for each class are present (HIGH and KMSKS for class I and motifs 1, 2 and 3 for class II).

Beside the conserved signature motifs and structure of class I AARS, the Rossmann fold is generally separated into two halves by the presence of two idiosyncratic sequences, named connective peptides 1 and 2 (CP1, CP2) [2]. Several functions have been described for CP1. Among them are editing of mischarged tRNA in LeuRS [3] and IleRS [4,5], although it is dispensable for the enzymatic activity [6], tRNA binding in GlnRS [7], species specificity between bacterial and eukaryal TyrRS [8], and zinc binding of cytoplasmic yeast MetRS [9]. To date, no function has been assigned to CP2 of class I AARS.

The crystal structure of *Bacillus stearothermophilus* TrpRS has been solved at a resolution of 1.7 Å [10,11]. TrpRS from the majority of the organisms of all three domains of life contains a relatively short CP2. TrpRS from the acidophilic bacterium *Acidithiobacillus ferrooxidans* contains an unusually long CP2. Similar or even longer CP2 domains are found in some euryarchaeal TrpRS proteins. Here we show that a deletion of 65 amino acids from the CP2 in *A. ferrooxidans* TrpRS results in an enzyme that retains in vitro and in vivo activity. Although the shortened TrpRS was fully active in tryptophan activation, kinetic analysis revealed that the K_m for cognate tRNA was seven-fold higher compared to the intact enzyme. Modeling of the tertiary structure of *A. ferrooxidans* TrpRS suggests that CP2 might interact with the tRNA, causing no distortion of the evolutionarily conserved structure of TrpRS.

2. Materials and methods

2.1. Cloning of trpS from A. ferrooxidans and Escherichia coli

A BLAST search [12] was used to identify the *trpS* gene in the genome sequence from *A. ferrooxidans* using the microbial database of The Institute for Genomic Research (http://www.tigr.org). The gene was amplified by PCR from genomic DNA from *A. ferrooxidans* ATCC 19859 using Elongase (Life Technologies) and the primers WSTF1 (5′-GAATTCTGAGAGAGATCATCGTTTC-3′) and WSTF2 (5′-GAATTCCGACGCGCCGGATTC3-′). The sequence recognized by *Eco*RI is underlined. The PCR product was cloned into pGEM-T (Promega). After digestion with *Eco*RI, the 1236 bp DNA was subcloned into *Eco*RI-digested pGEX-2T (Pharmacia). Plasmid pGWSAf-1 was obtained. *TrpS* from *E. coli* was also obtained by PCR amplification from genomic DNA, using the primers WSEC1 (5′-TATATAGGATCCTTAACGCTTC3-′) and WSEC2

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(5′-GAATTCTGACTAAGCCCATCG-3′). The DNA product was digested with *Bam*HI and *Eco*RI (underlined) and cloned into pGEX-2T, giving plasmid pTRS. Identity of *trpS* from pGWSAf-1 and pTRS was confirmed by DNA sequencing.

2.2. Deletion of the CP2 encoding segment from A. ferrooxidans trpS

To delete the region encoding 65 amino acids (residues 176–241) of the domain CP2 from *A. ferrooxidans trpS*, a modification of the PCR mutagenesis procedure was employed [13]. Using plasmid pGWSAf-1, a first PCR amplification of the DNA encoding the 5′ (primers WSTF1 and WSTF3, 5′-AGGATTTCATCGGCGTCGCG-3′) and 3′ (primers WSTF2 and WSTF4, 5′-ACGCCGATGAAATCCTTCGCGAA-3′) regions adjacent to the segment to be deleted was carried out. Two PCR products were obtained (539 and 520 bp respectively) which have an overlapping region (underlined in the sequence of primers WSTF3 and WSTF4). These PCR products were used as template in a second PCR amplification with the external primers WSTF1 and WSTF2, to obtain the *trpS* gene with the deletion in the CP2 domain. The deleted *trpS* (*trpSδ65*) was cloned into the *Eco*RI site of pGEX-2T giving the plasmid pGWSδ65. All DNA manipulations were confirmed by DNA sequencing.

2.3. Complementation of E. coli trp42c

E. coli strain *trp42c* [14], which carries a thermosensitive mutation in *trpS*, was transformed by electroporation with the desired plasmid (pGWSAf-1, pGWSδ65, pTRS or pGEX-2T), plated on LB ampicillin (100 μg/ml) and incubated for 16 h at 30°C. Isolated colonies were inoculated into 1 ml of LB ampicillin and cultivated for 16 h at 30°C. This culture was used to inoculate 10 ml of LB ampicillin to obtain an absorbance at 600 nm of 0.1 and then transferred to 40°C. Cell proliferation was monitored by measuring absorbance at 600 nm.

2.4. Preparation of A. ferrooxidans tRNA^{Trp}

The sequence of the *A. ferrooxidans* tRNA^{Trp} gene was obtained from The Institute of Genome Research Microbial Database. The gene was constructed by chemical synthesis (Centro de Síntesis y Análisis de Biomoléculas, Universidad de Chile) including *Eco*RI and *Hin*dIII sites at the 5′ and 3′ flanking regions respectively. The artificial gene was cloned into pKK223-3 (Amersham Pharmacia Biotech), generating the plasmid pKK-tRNA^{Trp}. The identity of the cloned gene was confirmed by DNA sequencing. *E. coli* DH5α was transformed by electroporation with the recombinant plasmid. Transformed cells were grown to an absorbance of 0.8 at 600 nm and the expression of the tRNA gene was initiated by the addition of 1 mM IPTG. The cells were further cultured for 3 h at 37°C. Purification of the tRNA from these cells was carried out according to the procedure described by Raczniack et al. [15]. Using glutathione *S*-transferase (GST)–TrpRS from *E. coli* as a source of TrpRS, the tRNA preparation aminoacylated 16.3 pmol of tryptophan per μg of total *E. coli* tRNA, compared to 0.057 pmol per μg of total tRNA obtained when tRNA from cells transformed with the empty vector was used.

2.5. In vitro enzymatic activity of recombinant TrpRSs

Recombinant TrpRS from *A. ferrooxidans* (GST–TrpRS and GST–TrpRSδ65) and *E. coli* (TrpRS) were expressed in *E. coli* JM105. Purification and enzymatic analyses of all TrpRSs fused to glutathione transferase were carried out as described previously [16].

3. Results

3.1. TrpRS sequence and modeling

A BLAST similarity search of the sequence of *B. stearothermophilus* TrpRS against the *A. ferrooxidans* genome sequence (The Institute of Genome Research Microbial Database) revealed an open reading frame encoding a 403 amino acids long protein. The predicted protein sequence aligns with TrpRS from different species. The encoded protein contains the signature motifs of class I AARS and a 70 amino acids long CP2 that is unusually long for TrpRS. When the sequence of the CP2 was excluded from the alignment, the highest score was obtained with *Thermotoga maritima* TrpRS (52% identity and 70% similarity). Neither evident promoter(s) nor ρ-independent transcription terminators were found 400 nucleotides upstream or downstream of the *trpS* gene respectively.

Alignment of TrpRS sequences encoded in the genome of organisms from all three phylogenetic domains revealed that the CP2 is not present in any other bacterial or eukaryal TrpRS (Fig. 1). However, the predicted sequence of some archaeal TrpRS of Euryarchaeota also contain a CP2 (59–129 amino acids long) in the same relative position as in *A. ferrooxidans* (based on the predicted protein sequence alignment). At the amino acid level, these putative CP2s have low sequence similarity (data not shown).

The structure of *B. stearothermophilus* TrpRS has been solved at 1.7 Å resolution. This is a dimer of two identical subunits [11]. Based on the similarity of amino acid sequence, *B. stearothermophilus* TrpRS was used as template to model the three-dimensional structure of *A. ferrooxidans* TrpRS. In order to model properly, the sequence of the putative CP2 was excluded from the procedure. The predicted three-dimensional structure has an root mean square deviation of 0.58 Å compared to *B. stearothermophilus* TrpRS (data not shown). Residues which are known to interact with the intermediate tryptophanyl-AMP in *B. stearothermophilus* have similar distances in the model. Secondary structure analysis revealed that 83% of amino acids present favorable ϕ and ψ angles. These observations support the prediction that the structure of *A. ferrooxidans* TrpRS might be similar to *B. stearothermophilus* TrpRS (data not shown). Based on the predicted

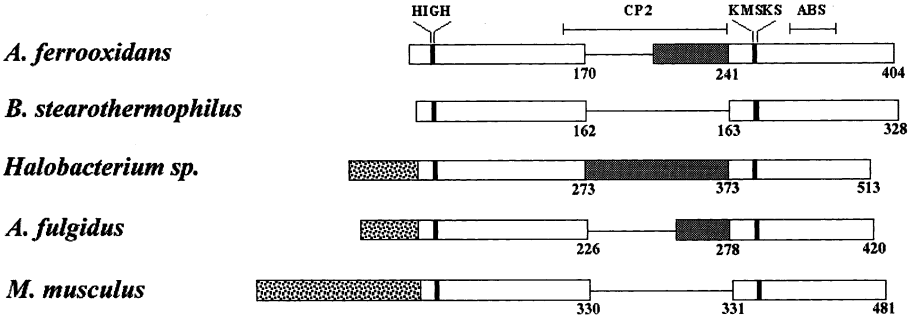

Fig. 1. Schematic representation of the alignment of TrpRSs sequences from organisms representative of the three domains of life. Conserved regions (white), CP2 (black) and extensions at the amino-terminus of archaeal and eukaryal TrpRS (stippled) are represented. Thin lines represent the connection between contiguous sequences. All TrpRS are aligned based on the HIGH and KMSKS signature motifs. Anticodon binding site (ABS) and CP2 relative positions are indicated.

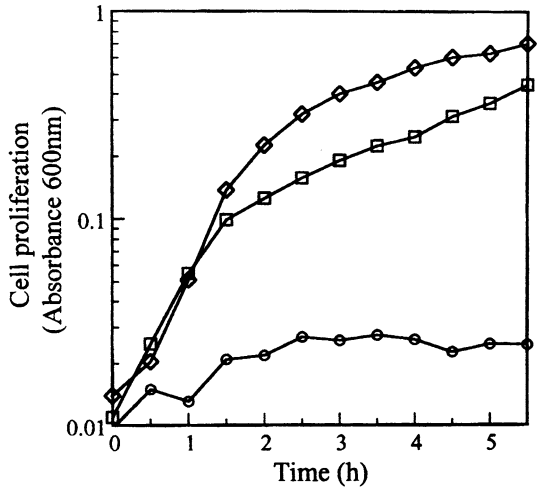

Fig. 2. Complementation of *E. coli trp42c* with *A. ferrooxidans trpS* variants. *E. coli* thermosensitive mutant cells were transformed with plasmids carrying *A. ferrooxidans trpS* wild type (◇) or δ*65* (□) genes fused to GST. Cells were grown at the restrictive temperature and proliferation was monitored by absorbance at 600 nm. ○, empty vector.

structure, CP2 is localized near the interface of the two subunits of the dimer.

3.2. CP2 is dispensable for A. ferrooxidans TrpRS activity

To further biochemically and genetically characterize TrpRS, the gene encoding *A. ferrooxidans* TrpRS was cloned in pGEX-2T, which allows the expression of TrpRS fused to GST under the control of isopropyl-β-galactoside.

A deletion of the DNA segment encoding residues 176–241 of TrpRS (65 out of 70 residues of CP2) was carried out. Functionality of recombinant wild type (*trpS*) and deleted (*trpS*δ*65*) genes was tested by rescuing the thermosensitive phenotype of *E. coli trp42c*. Both wild type *A. ferrooxidans trpS* and *trpS*δ*65* successfully suppressed the mutation at the non-permissive temperature (Fig. 2). These results indicate that *A. ferrooxidans* TrpRS can recognize *E. coli* tRNA and that CP2 is not essential for the in vivo activity of the enzyme in *E. coli*.

3.3. Is CP2 from A. ferrooxidans TrpRS involved in the aminoacylation of tRNA?

To further characterize the biochemical properties of recombinant *A. ferrooxidans* GST–TrpRS and GST–TrpRSδ65, protein products were overexpressed in *E. coli* and purified by affinity chromatography on glutathione-agarose. As both fusion products were mostly insoluble even when the cells were cultivated at 25°C, the yield in the purification procedure was only 0.028 mg and 0.024 mg per liter of culture for GST–TrpRS and GST–TrpRSδ65 respectively. Recombinant *E. coli trpS*, cloned in the same DNA vector, yielded 7.6 mg of the fusion product GST–TrpRS per liter of culture.

Both GST–TrpRS and GST–TrpRSδ65 activate tryptophan practically at the same rate, as measured by the PPi exchange assay (Table 1). While the deletion causes no changed in the K_m for tryptophan, K_m for ATP and k_{cat} are slightly altered. As the kinetic parameters of the activation reaction are similar in the wild type and the deleted variant of TrpRS, we assume that the structure of the activation domain is not modified upon deletion of CP2. Activation of 5-fluorotryptophan, an analog that has been described as more hydrophobic than tryptophan [17], was also not affected by the deletion on *A. ferrooxidans* TrpRS (data not shown), supporting that the deletion caused no effect on the active site of the enzyme. The effect of the fusion of GST to TrpRS was analyzed in *E. coli* TrpRS fused to GST. Kinetic parameters of recombinant *E. coli* TrpRS were similar to the reported values of the wild type enzyme (Table 1) [14], suggesting that the fusion to glutathione transferase has no effect on the catalytic properties of the enzymes.

A total tRNA preparation from *E. coli* that overexpressed *A. ferrooxidans* $tRNA^{Trp}$ was used as substrate for the aminoacylation reaction. Kinetic analysis of the aminoacylation of $tRNA^{Trp}$ revealed that TrpRSδ65 has a K_m which is sevenfold higher compared to wild type TrpRS (Table 1). A slight decrease in k_{cat} of aminoacylation led to a 10-fold decrease in the catalytic efficiency of the overall reaction. These results imply that the CP2 from *A. ferrooxidans* TrpRS might participate in the binding of tRNA to the enzyme. Whether there is a direct effect of CP2 on the binding of $tRNA^{Trp}$ or the observed increase in K_m is due to a global change in the protein structure upon the deletion is yet to be elucidated.

4. Discussion

The presence of a remarkably long CP2 in *A. ferrooxidans* TrpRS as well as in some members of the Euryarchaeota in the same relative position (although with low, if any, sequence similarity) might indicate that the CP2 present in contemporary TrpRS is a remnant of CP2 from ancient TrpRS in the process of disappearance. Modeling of the tertiary structure of *A. ferrooxidans* TrpRS was possible only when amino acids of CP2 were excluded from the query sequence. The predicted structure was found to be similar to *B. stearothermophilus* TrpRS. It suggests that CP2 is an appendage on the surface of the protein and the rest of the enzyme can acquire the overall conserved structure. *A. ferrooxidans* TrpRS is active in vivo in *E. coli*. It implies that *A. ferrooxidans* TrpRS utilizes *E. coli* $tRNA^{Trp}$ as substrate. Deletion of CP2 has no evident effect on the function of the expressed protein in vivo in *E. coli*. In vitro kinetic analysis revealed that neither K_m of

Table 1
Kinetic parameters of recombinant TrpRS

Enzyme	Activation of tryptophan				Aminoacylation of $tRNA^{Trp}$		
	K_m Trp (μM)	K_m ATP (μM)	k_{cat} (s^{-1})	k_{cat}/K_m Trp (s^{-1} μM^{-1})	K_m $tRNA^{Trp}$ (μM)	k_{cat} (s^{-1})	k_{cat}/K_m $tRNA^{Trp}$ (s^{-1} μM^{-1})
A. ferrooxidans TrpRS wt	1.45	128.6	24.5	16.9	0.31	3.17	10.2
A. ferrooxidans TrpRSδ65	1.87	252.2	13.6	7.3	2.02	2.22	1.1
E. coli TrpRS	16.81	–	150.6	9.0	0.62	13.9	22.4

tryptophan and ATP nor k_{cat} for the activation of tryptophan is substantially affected by the deletion of CP2, suggesting that the conformation of the activation domain of TrpRS might not be distorted by the deletion. In contrast, a considerable increase in the K_m of the enzyme for the cognate tRNA was evidenced, with the concomitant effect in the efficiency of the aminoacylation reaction. The activation domain of class I synthetases interacts with the acceptor stem of tRNA [7], which carries the discriminator base located adjacent to the CCA of tRNA. It seems to be the case with *Bacillus subtilis* TrpRS as well [18]. Based on the model of *A. ferrooxidans* TrpRS described here and the model of *B. subtilis* TrpRS–$tRNA^{Trp}$ complex, proposed by Jia et al. [18], we believe that CP2 is localized close to the inner portion of the L shape of tRNA. From these assumptions, we hypothesize that CP2 might contribute to facilitate the interaction of *A. ferrooxidans* TrpRS with $tRNA^{Trp}$.

The observations described in this report raise the question whether there is any other role of *A. ferrooxidans* TrpRS in addition to aminoacylation of $tRNA^{Trp}$. Archaeal $tRNA^{Trp}$ genes contain introns which have to be removed from the primary transcript RNAs to yield active substrates for TrpRS [19]. Whether the CP2 from archaeal TrpRS might have a function in the processing of the tRNA is an alternative to be investigated. It is interesting to note that BLAST searches of *A. ferrooxidans* TrpRS CP2 to protein sequence databases revealed similarity to a portion of the Rossmann fold of certain dehydrogenases (data not shown). It has been described that glyceraldehyde-3-phosphate dehydrogenase from human cells [20] has, among several other, a specific tRNA binding activity. NAD inhibited the tRNA binding suggesting that the Rossmann fold is involved in this activity. Whether the long CP2 of *A. ferrooxidans* and archaeal TrpRS plays a role in functions not yet discovered other than protein synthesis is an open question.

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