Substrate-induced conformational changes in *Escherichia coli* arginyl–tRNA synthetase observed by ^{19}F NMR spectroscopy

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Abstract The ^{19}F nuclear magnetic resonance (NMR) spectra of 4-fluorotryptophan (4-F-Trp)-labeled *Escherichia coli* arginyl–tRNA synthetase (ArgRS) show that there are distinct conformational changes in the catalytic core and tRNA anticodon stem and loop-binding domain of the enzyme, when arginine and $tRNA^{Arg}$ are added to the unliganded enzyme. We have assigned five fluorine resonances of 4-F-Trp residues (162, 172, 228, 349 and 446) in the spectrum of the fluorinated enzyme by site-directed mutagenesis. The local conformational changes of *E. coli* ArgRS induced by its substrates observed herein by ^{19}F NMR are similar to those of crystalline yeast homologous enzyme.

Key words: Aminoacyl–tRNA synthetase; $tRNA_2^{Arg}$; Arginine; ATP; 4-fluorotryptophan; *Escherichia coli*

1. Introduction

Aminoacyl-tRNA synthetases (aaRSs) catalyze the esterification of amino acids to the 2′- or 3′-hydroxyl group of ribose at the 3′ end of their cognate tRNAs, and play a key role in protein biosynthesis in vivo [1]. The majority of aaRSs catalyze the aminoacylation of their cognate tRNAs in a two-step reaction comprising amino acid activation and a tRNA aminoacylation reaction [1].

Escherichia coli arginyl-tRNA synthetase (*E. coli* ArgRS, EC 6.1.1.19) belongs to class I aaRSs that are characterized by the structural motifs HIGH and KMSKS [2]. *E. coli* ArgRS, of which the molecular mass is theoretically 64.678 kDa, consists of 577 amino acid residues [2]. ArgRS, as well as the glutamyl–tRNA synthetase and glutaminyl–tRNA synthetase, requires its cognate tRNA to catalyze the amino acid activation reaction, which is different from the other 17 aaRSs [3–9]. A full understanding of such abnormal catalytic mechanisms requires structural information. The crystal structure of *E. coli* ArgRS is not yet known, although its crystallization has been accomplished [10]. Therefore, to probe the subtle changes caused by substrates of *E. coli* ArgRS in solutions and further clarify the catalytic mechanism of ArgRS, we performed ^{19}F nuclear magnetic resonance (NMR) spectroscopy of *E. coli* ArgRS labeled with 4-fluorotryptophan (4-F-Trp) in the absence and presence of its substrates: arginine, $tRNA_2^{Arg}$ (transfer RNA isoacceptor for arginine(ACG)), and ATP. For proteins too large or unstable for full NMR structural determination, ^{19}F NMR spectroscopy provides valuable information about conformational changes. The chemical shift value of ^{19}F nucleus that is readily introduced into protein is highly sensitive to the change of its environment, making it an excellent probe for studying protein conformational changes [11–13].

The biosynthetic incorporation of 4-F-Trp in *E. coli* ArgRS has shown that the fluorine had little effect on the function and structure of this enzyme [14]. Five Trp residues (162, 172, 228, 349 and 446) are found in *E. coli* ArgRS [9,14]. The amino acid sequence of *E. coli* ArgRS is 28.7% identical to that of yeast homolog enzyme [5]. Five Trp residues of *E. coli* ArgRS are located in interesting and crucial regions, of which conformations may be altered upon the substrate binding (yeast ArgRS structure; Fig. 1) [5,7]. The catalytic core for both amino acid activation and tRNA aminoacylation reactions in ArgRS, which forms the scaffold for the Rossmann fold, is composed of two halves assembled from three peptides [5,7]. The first half of the catalytic core contains two peptides – Lys143–Lys194 and Trp266–Gly293 – while the second half includes residues Thr345–Thr410 in yeast ArgRS [5,7]. Trp162 (yeast Trp192) and Trp228 (yeast Trp266) residues are located at the edge of the first half of the catalytic core, while Trp349 (yeast Phe383) residue is in the second half. Trp446 (yeast Trp475) is in the proximity of a tRNA anticodon stem binding element (a so-called Ω loop from Ser480 to Thr485 in yeast). Trp172 (yeast Gly202) residue is buried in the Ins-1 (yeast Gln195–Ile265) domain that inserts itself into the catalytic core [5,7]. Therefore, it is possible to probe the conformational changes involving these regions in solution with these probes. In this study, we produced 4-F-Trp-labeled *E. coli* ArgRS (FWT) and five site-directed mutants (Trp to Ala), and our assignment of five NMR signals in the spectrum of FWT to these specific Trp residues allowed us to study *E. coli* ArgRS conformational changes caused by substrate binding.

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Abbreviations: aaRS, aminoacyl-tRNA synthetase; ArgRS, arginyl-tRNA synthetase; 4-F-Trp, 4-fluorotryptophan; FWT, 4-F-Trp-labeled *E. coli* ArgRS; $tRNA_2^{Arg}$, transfer RNA isoacceptor for arginine(ACG)

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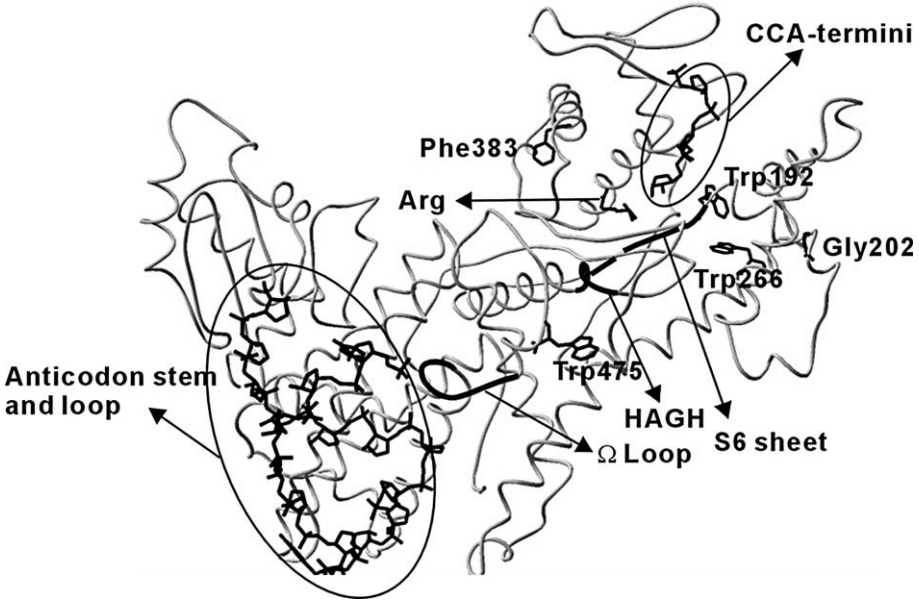

Fig. 1. Structure of yeast ArgRS liganded with arginine and its cognate tRNA [7]. The figure shows the α-carbon backbone of the enzyme and partial phosphate-ribose backbone of tRNA. Crucial structural elements are labeled, including S6 sheet, Ω loop, and the characteristic sequence HIGH (HAGH in yeast ArgRS) for Class I aaRSs. The five residues aligned to five Trp residues in *E. coli* ArgRS are highlighted with their sidechains. In the alignments of ArgRS from 17 species varying from *Proteobacteria* to *Eukaryota* (data not shown), *E. coli* Trp162 (yeast Trp192) and *E. coli* Trp228 (yeast Trp266) are conserved as Trp residues, and *E. coli* Trp349 (yeast Phe383) and *E. coli* Trp446 (yeast Trp475) are conserved as aromatic residues. *E. coli* Trp172 is not conserved.

2. Materials and methods

2.1. Materials

All chemicals except where noted were purchased from Sigma. All enzymes used in DNA manipulations were purchased from Biolabs (New England). The expressing plasmid was pTrc99B, gifted by Prof. J. Gangloff (CNRS, France), and the tryptophan auxotrophy strain was W3100 trpA, gifted by Prof. H. Xue (HKUST, Hong Kong). $tRNA_2^{Arg}$, used in ^{19}F NMR titration into FWT, was isolated in our laboratory [15].

2.2. Production of FWT and its mutants

The genes (*args*) encoding for wild-type *E. coli* ArgRS and its five Trp-to-Ala mutants W162A, W172A, W228A, W349A, and W172&228A were recombined into the *Nco*I and *Hin*dIII sites of pTrc99B and transformed into *E. coli* tryptophan auxotrophy W3110 trpA33, according to the method described previously [16]. All recombinant plasmids were confirmed by DNA sequencing. In W3110 trpA33, the ArgRS and its mutants incorporated with 4-F-Trp were overproduced by induction with 1-isopropyl-β-D-1-thiogalactopyranoside and then purified according to the method described previously [14]. The percentage of 4-F-Trp incorporation in the 4-F-Trp-labeled *E. coli* ArgRS (FWT) and mutants (FW162A, FW172A, FW228A, FW349A, and FW172&228A) was determined as described elsewhere [14]. The protein concentration was determined by the Lowry method [17]. Before the ^{19}F NMR measurements, all protein samples were dialyzed three times against buffer A (50 mM Tris–HCl, pH 7.5, 80 mM KCl, 8 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol) and concentrated to 0.5 mM with Centricon-50 (Millipore).

2.3. ^{19}F NMR measurements

^{19}F NMR spectra were obtained at 564.277 MHz on a Varian Unity INOVA 600 spectrometer, using a $^{19}F/^{1}H$ [BB] probe (Nalorac). Samples containing 4-F-Trp-labeled proteins (0.5 mM) in buffer A were prepared by adding 5% (v/v) D_2O as the lock solvent. 4-F-Trp was used as an external chemical shift reference at 0 ppm. Measurement parameters included a spectral width of 10 000 Hz, 8192 data points, a 1.0 s relaxation delay, a temperature of 35°C, and spectral processing with 20 Hz line broadening in the mutants' spectra and 10 Hz broadening in the titration spectra. The line widths of fluorine resonances in the ^{19}F NMR spectra obtained in the experiment ranged from 280 to 350 Hz, which were comparable to those in the ^{19}F NMR spectra of other, similar molecular mass proteins [18].

3. Results and discussion

3.1. Production of FWT and its mutants

From 2 l culture, 20–80 mg of FWT and its mutants with a homogeneity of over 90% was obtained. Although the incorporation extent of 4-F-Trp into FWT and its mutants varies (Table 1), the extent of labeling is sufficient for ^{19}F NMR spectroscopy. Depending on different proteins and applications, the incorporation efficiency of 4-F-Trp in proteins frequently varies from less than 5% to more than 90% in previous studies [11]. In addition, FWT with 95% incorporation of 4-F-Trp has been determined with 80% aminoacylation activity of the native ArgRS [14]. The FW162A that will be used in the assignment was determined to retain 33.7% of activity compared with FWT, using methods described previously [3,14].

3.2. Assigning the FWT spectrum

The ^{19}F NMR spectrum of FWT is compared with the individual spectra of the fluorinated mutants (Fig. 2). Observing which peak in the FWT spectrum is eliminated in the

Table 1
Efficiency of 4-F-Trp incorporation[a]

Protein	FWT	FW162A	FW172A	FW228A	FW172&228A	FW349A
4-F-Trp incorporation	95%	60%	43%	74%	20%	47%

[a]The determinations of incorporation efficiency are described in Section 2.2.

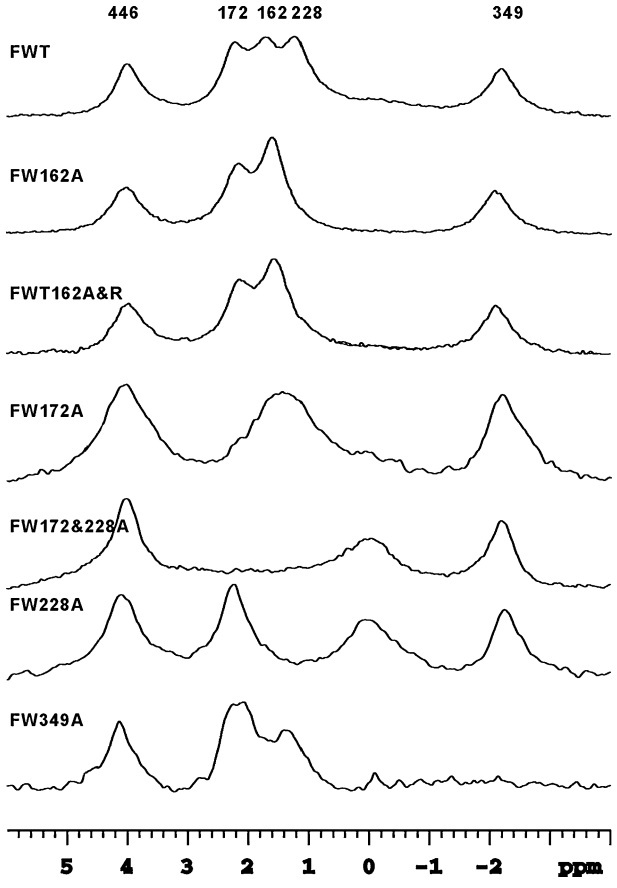

Fig. 2. The asssignment of fluorine resonances in FWT by site-directed mutagenesis. Shown are the wild-type and the mutants of *E. coli* ArgRS labeled with 4-F-Trp (FWT, FW162A, FW172A, FW172&228A, FW349A). FW162A&R represents FW162A titrated with 0.5 mM arginine. All samples contain buffer A, 5% D_2O, and 0.5 mM protein. ^{19}F NMR spectra were recorded as described in Section 2.3.

spectrum of each mutant leads to the assignment of 4-F-Trp resonances in the ^{19}F NMR spectrum. The resonances of 4-F-Trp172, 4-F-Trp349, and 4-F-Trp446 are readily identified by comparing the remaining or eliminating the resonances in the corresponding mutants' spectra and those in the FWT spectrum. FW162A is able to bind arginine as well as exhibit aminoacylation activity. Due to the arginine binding, the resonance at 1.7 ppm is altered in the spectrum of FWT (Fig. 3B), but this alteration disappears in the arginine titration spectrum of FW162A (FW162A, FW162A&R in Fig. 2). Therefore, the arginine-induced and alterable resonance at 1.7 ppm in the FWT spectrum can be assigned to 4-F-Trp162. Finally, the unassigned resonance at 1.2 ppm is assigned to 4-F-Trp228. The resonances for 4-F-Trp162, 4-F-Trp172, and 4-F-Trp228 overlap significantly in the FWT spectrum, indicating that these three residues exhibit a similar chemical environment. Additionally, these three residues are near each other in yeast ArgRS, and especially yeast homolog residues of Trp162 and Trp228 almost osculate (Fig. 1).

3.3. *Effects of Trp-to-Ala mutations on ^{19}F NMR chemical shifts*

The overall ^{19}F NMR spectra of the mutants are similar to that of FWT, except that the fluorine resonance associated with the missing Trp is absent. The fluorine resonances of 4-F-Trp349 and 4-F-Trp446 are unaffected by the replacement of the other three Trp residues in their corresponding mutants' spectra (Fig. 2). This may result from the fact that Trp349 and Trp446 are distant from the other three Trp residues (Fig. 1). However, Trp-to-Ala substitution in one of the 162 and 228 positions causes perturbations in the spectra of FW162A and FW228A (Fig. 2). In the catalytic core, Trp162 is located in a flexible S6 β-sheet that backs onto a tight α-helix where Trp228 is located (Fig. 1). This may explain why Ala substitution of Trp228 has a larger effect on the resonance frequency of 4-F-Trp162 than does the Trp162-to-Ala substitution on that of 4-F-Trp228 (Fig. 2, FW228A and FW162A). None of the substitutions affects the fluorine resonance of 4-F-Trp172, but the fluorine resonances of 4-F-Trp162 and 4-F-Trp228 are affected by the replacement of Trp172 to Ala (Fig. 2, FW172A). Trp172 is buried in an Ins-1 domain (Fig. 2), so the reduction of the Trp172 side-chain may significantly affect the inner structure of this domain, whereas the effect on the local structure of Trp162 and Trp228 is over a longer distance. Similar perturbations of fluorine signals induced by Trp substitution have been previously observed in tissue factor [19], glucose and galactose binding protein [20], and leucine-specific binding protein [21].

3.4. *Effects of arginine, $tRNA^{Arg}$, and ATP on the conformation of* E. coli *ArgRS*

The above assignments enable the five 4-F-Trp resonances to be used as probes of the local conformational changes to the five Trp positions within the *E. coli* ArgRS. The titrations of arginine, $tRNA^{Arg}$, and ATP into FWT are shown in Fig. 3. As determined previously, the binding constants of *E. coli*

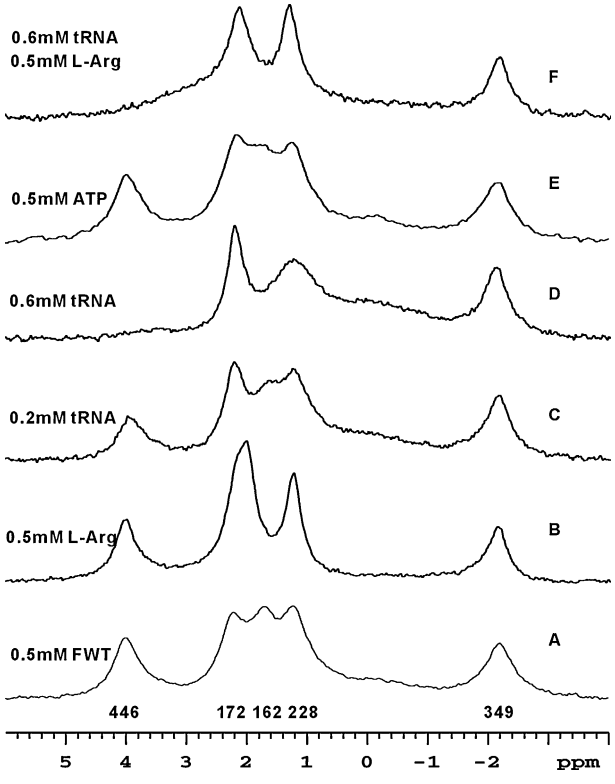

Fig. 3. Effects of substrate binding on the ^{19}F NMR of FWT. Shown are the spectra of 0.5 mM FWT titrated with its substrates. The substrates and their concentrations are indicated. Experimental conditions are the same as in Fig. 2.

ArgRS for arginine, ATP, and $tRNA^{Arg}$ are 70 μM, 0.75 mM, and 0.45 μM, respectively [22].

Fig. 3B shows the effects of adding stoichiometric amounts of arginine into FWT on the resonance of 4-F-Trp162. These results show that the environment of Trp162 is changed due to the binding of arginine in the first half of the catalytic core of *E. coli* ArgRS. Our previous mutation result shows that the Trp162-to-Ala mutant exhibits lower catalytic activity than the native enzyme [23]. Through comparison of yeast ArgRS–tRNA and ArgRS–tRNA–arginine structures, it is found that arginine binding to tRNA-liganded yeast ArgRS will destroy a hydrogen bond between Tyr347 (*E. coli* Try313) and Trp192 (*E. coli* Trp162) [5,7]. ^{19}F NMR data at present illustrate that the 4-F-Trp162 resonance frequency is affected by the binding of arginine to tRNA-free *E. coli* ArgRS, therefore suggesting that the local structure of Trp162 is involved in arginine binding.

E. coli $tRNA_2^{Arg}$ is selected for the $tRNA^{Arg}$ titration, because it is the most frequently used isoacceptor tRNA for arginine in *E. coli* [24]. The binding of $tRNA^{Arg}$ to FWT causes large changes in the ^{19}F NMR spectra, with only the resonances of 4-F-Trp172 and 4-F-Trp349 being unaffected (Fig. 3C,D). The resonance frequency of 4-F-Trp446 is greatly affected upon binding of $tRNA^{Arg}$. In yeast ArgRS, the Trp475 residue (*E. coli* Trp447) is followed by a crucial element for the positioning of the tRNA anticodon stem, an Ω loop, whose conformation is markedly altered by yeast cognate tRNA binding [7]. Yeast Trp475 residue (*E. coli* Trp446) also belongs to a helix element-H17 (yeast Trp475 to Leu479) that participates in tRNA anticodon loop binding [7]. Here, in ^{19}F NMR, the change of 4-F-Trp446 resonance frequency suggests that the local structure of Trp446 in *E. coli* ArgRS is also involved in the binding of $tRNA^{Arg}$. Other altered resonances include those of 4-F-Trp162 and 4-F-Trp228, of which yeast homolog residues are present at the catalytic core. We surmise that the local conformational alteration of 4-F-Trp162 and 4-F-Trp228 is due to the interaction between the acceptor stem of $tRNA^{Arg}$ and the catalytic core of the enzyme. The *E. coli* $tRNA^{Arg}$ identity element includes A/G73, C35, U/G36 and A20 [25]. It is therefore reasonable that *E. coli* $tRNA^{Arg}$ mainly interacts with *E. coli* ArgRS by its acceptor stem (including the CCA terminal) and anticodon stem and loop, and thereby causes conformational changes of the two $tRNA^{Arg}$ binding domains in *E. coli* ArgRS-catalytic core, and the anticodon stem and loop-binding domain.

The addition of ATP does not affect any of the resonances of the five 4-F-Trp residues in the FWT spectrum here (Fig. 3E). We also performed ^{19}F NMR titration of ATP with 2:1 mol ratio (ATP:FWT) into arginine or tRNA-bound FWT, but ATP binding had no effect (data not shown). These results may be due to the large distance between the predicted ATP binding site (yeast His159-Ala160-Gly161-His162, HAGH) and the five fluorine probes (4-F-Trp residues) (Fig. 1) [5,7].

3.5. *Integral structural effects of arginine and tRNA on* E. coli *ArgRS*

The spectrum of arginine and $tRNA_2^{Arg}$-bound *E. coli* ArgRS is shown in Fig. 3F. Large differences are evident between Fig. 3F and Fig. 3D. When arginine and $tRNA^{Arg}$ bind together to *E. coli* ArgRS, the alteration to the catalytic core near Trp162 and Trp228 is different from that induced by $tRNA^{Arg}$ binding alone. As described above, arginine binding in yeast ArgRS prevents the formation of a hydrogen bond between Tyr347 and Trp192 (*E. coli* Tyr313 and Trp162), and then Tyr347 is free to participate in the assembly of the CCA terminal of tRNA in the catalytic core [5,7]. The arginine-induced changes on the ^{19}F NMR spectrum of $tRNA_2^{Arg}$-bound FWT may have a similar reason as for the hydrogen bond between *E. coli* Tyr313 and Trp162 residues. Additionally, ATP does not affect the spectrum of FWT bound with arginine and tRNA (data not shown), possibly due to the large distance between the five fluorine probes and the ATP binding site as described above.

Arginine and/or $tRNA^{Arg}$ induce(s) some distinct local conformational changes in the catalytic core; additionally, $tRNA^{Arg}$ interacts with the anticodon stem and loop-binding domain of *E. coli* ArgRS. These ^{19}F NMR results on the local conformational changes of *E. coli* ArgRS catalytic core and anticodon stem and loop-binding domain are consistent with the previous results of yeast ArgRS co-crystal structure with arginine and yeast $tRNA^{Arg}$ [5,7].

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