J. Biochem. Mol. Biol. Vol. 28, No. 4 pp. 363~367 July 31, 1995

Quantitative Analysis of Protein-RNA Interaction in A Class I tRNA Synthetase by Saturation Mutagenesis

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(Received April 1, 1995)

Abstract : *E. coli* methionyl-tRNA synthetase is one of the class I tRNA synthetases. The Tryptophane residue at the position 461 located in the C-terminal domain of the enzyme is a key amino acid for the interaction with the anticodon of $tRNA^{Met}$. W461 was replaced with other amino acids to determine the chemical requirement for the interaction with the anticodon of $tRNA^{Met}$. Saturation mutagenesis at the position 461 generated a total of 12 substitution mutants of methionyl-tRNA synthetase. All the mutants showed the same *in vivo* stability as the wild-type enzyme, suggesting that the amino acid substitutions did not cause severe conformational change of the protein. The mutants containing tyrosine, phenylalanine, histidine and cysteine substitutions showed *in vivo* activity while all the other mutants did not. The comparison of the *in vitro* aminoacylation activities of these mutants showed that aromatic ring structure, Van der Waals volume and hydrogen bond potential of the amino acid residue at the position 461 are the major determinants for the interaction with the anticodon of $tRNA^{Met}$.

Key words : aminoacylation, methionyl-tRNA synthetase, protein-RNA interaction, saturation mutagenesis.

Aminoacyl-tRNA synthetases catalyze the attachment of amino acids to the 3′ end of their cognate tRNAs. These enzymes are classified into two groups depending on the conserved sequence motifs, although they are all involved in the same type of chemical reaction (Burbaum and Schimmel, 1991; Cusack *et al.*, 1991; Eriani *et al.*, 1990; Nagel and Doolittle, 1991). The class I synthetases contain signature sequences such as HIGH (Ludmerer and Schimmel, 1987; Webster *et al.*, 1984) and KMSKS (Hountondji *et al.*, 1986) in their catalytic domain, while the class II enzymes share degenerate sequence motifs at three regions along the protein sequences. Each aminoacyl-tRNA synthetase recognizes the cognate tRNA in a unique fashion, thereby avoiding a false interaction with non-cognate tRNAs, which could cause a critical defect in protein synthesis.

E. coli methionyl-tRNA synthetase is a class I aminoacyl-tRNA synthetase. The native form is a homodimer of 676 amino acid protomers (Dardel *et al.*, 1984) and the monomer enzyme with approximately 100 amino acid C-terminal truncation retains the full enzyme activity (Cassio and Waller, 1971). The X-ray crystal structure of the active monomeric enzyme showed that the protein consists of two domains which are distinct in their structure and function (Brunie *et al.*, 1987; Brunie *et al.*, 1990; Zelwer *et al.*, 1982) (Fig. 1). The N-terminal domain contains a nucleotide-binding fold and is involved in methionine activation and transferring the activated methionine to the acceptor end of the bound $tRNA^{Met}$ (Hountondji *et al.*, 1990). The C-terminal domain is mainly α-helical and makes a major

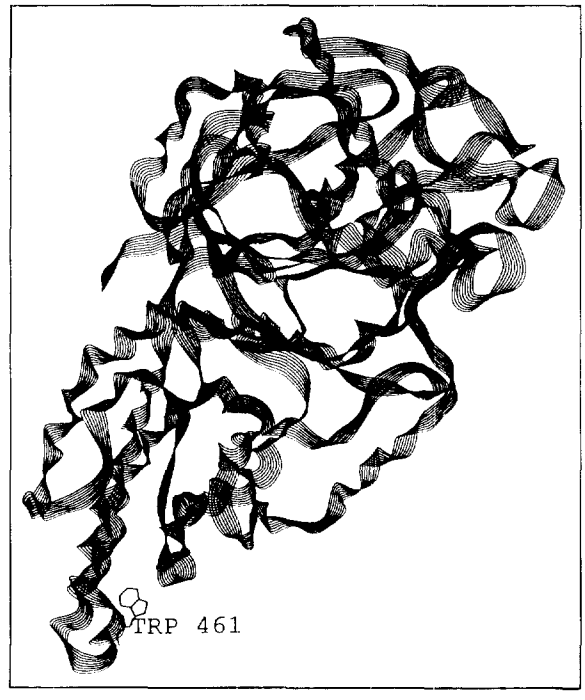

Fig. 1. Ribbon structure of *E. coli* methionyl-tRNA synthetase. W461 in the C-terminal domain is labelled.

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interaction with the anticodon of $tRNA^{Met}$ (Ghosh *et al.*, 1990; Perona *et al.*, 1991; Valenzuela and Schulman, 1986).

W461 of *E. coli* methionyl-tRNA synthetase is located at the junction between the surface α-helix and the loop in the C-terminal domain (Fig. 1). Although it is known that this residue is essential for the interaction with the anticodon of $tRNA^{Met}$ (Ghosh *et al.*, 1990), it is not clearly understood how this residue achieves its specific interaction with the anticodon of $tRNA^{Met}$. To understand the molecular mechanism of the interaction between W461 and the anticodon of $tRNA^{Met}$, saturation mutagenesis was made at the position 461. The ability of the substitution mutants at the position 461 to interact with the anticodon of $tRNA^{Met}$ was tested by the aminoacylation activities *in vivo* and *in vitro*.

Materials and Methods

Saturation mutagenesis at position 461

Phagemid pJB104 contains a gene encoding the monomeric *E. coli* methionyl-tRNA synthetase of 547 amino acids (Kim and Schimmel, 1992). Single stranded DNA was prepared by infecting *E. coli* JM109 harboring pJB104 with the helper phage M13KO7 (Pharmacia). A mutagenic oligonucleotide with a sequence of TTTCGCCACCAC(C/GNN)CGGAGCCTGTTTC (C/G represents 50% mixture of C and G; N represents a 25% mixture of A, T, G and C) was synthesized and annealed to the single stranded DNA template. Double stranded phagemid was made following the manufacturer's instruction for oligonucleotide-directed mutagenesis (Amersham). The phagemids were randomly isolated from the ampicillin resistant transformants and the substitution mutations at the position 461 were confirmed by DNA sequencing.

In vivo stability of mutants

Proteins that are not properly folded should be susceptible to proteolysis in the cell. To determine the effect of the mutations on protein structure, *in vivo* stability of the various mutant proteins was compared in *E. coli* strain TG1[F'*traD36 lacI*^{q}Δ(*lacZ*)*M15proA*$^+$ *B*$^+$/*supE*Δ(*hsdM-mcrB*)*5*($r_k^- m_k^-$ *McrB*$^-$)*thi*Δ(*lac-proAB*)] by immunoblotting of each mutant with rabbit antiserum raised against *E. coli* methionyl-tRNA synthetase (Kim and Schimmel, 1992). Cells expressing each substitution mutant were harvested and the total cellular proteins were resolved on 8% SDS-polyacrylamide gel. The proteins separated on the gel were transferred onto Immobilon membrane (Millipore) and each mutant methionyl-tRNA synthetase was bound to rabbit antibody. The enzyme-antibody complex on the membrane was detected using a chemiluminesence system (ECL, Amersham).

In vivo activity of mutants

To investigate the *in vivo* activities of the mutants, the phagemid expressing each mutant enzyme was transformed into *E. coli MetG* null strain, which lacks the chromosomal copy of *MetG* encoding methionyl-tRNA synthetase. This strain can maintain its viability at 30℃ with the help of another copy of *MetG* gene cloned in plasmid (pRMS615) (Kim *et al.*, 1993). This plasmid (called maintenance plasmid) contains temperature-sensitive replicon and thus cannot replicate at 42℃. Therefore, the activities of the mutants can be tested in this host strain by their ability to support cell growth at 42℃.

Aminoacylation assay and determination of relative enzyme activity

Cells expressing the mutant enzymes were lysed by ultrasonication and the crude cell extracts were prepared after removing cell debris by centrifugation. The relative aminoacylation activities of the mutant enzymes were compared with that of the wild-type enzyme following the previously described method (Kim *et al.*, 1993). The reaction was carried out in 20 mM HEPES buffer (pH 7.5) containing 4 mM $MgCl_2$, 100 μM EDTA, 150 mM NH_4Cl and 0.1 mg/ml bovine serum albumin. As reaction substrates, 4 μM $tRNA^{fMet}$ and 20 μM [^{35}S]labeled methionine were added. Aliquots of the reaction were taken at 1 min time intervals and the reaction in each aliquot was stopped on the glass fiber filter pads (2.3 cm diameter, Whatman) which had been presoaked with 5% trichloroacetic acid containing 1 mM methionine. The filter pads were washed in ice-cold 5% trichloroacetic acid with 1 mM methionine and dried by washing with diethylether and IR irradiation. The [^{35}S]methionine charged to $tRNA^{fMet}$ will have remained on the filter pad and it can be quantified by scintillation counting. The reaction rate shown by each mutant was normalized by the amount of the enzyme which was determined by immunoblotting.

Results and Discussion

The specific molecular interactions have been shown in a few complexes of tRNA synthetase and their cognate tRNAs (Biou *et al.*, 1994; Podjarny *et al.*, 1991; Rould *et al.*, 1989). These structural and other biochemical data suggest that the pattern of cognate tRNA recognition by aminoacyl-tRNA synthetases varies depending on the type of the enzyme (Shimmel, 1989). While *E. coli* alanyl-tRNA synthetase is one extreme

that makes a major interaction with a base pair in the acceptor stem of $tRNA^{Ala}$ (Hou and Schimmel, 1988; Park and Schimmel, 1988), methionyl-tRNA synthetase is the other that mainly recognizes the anticodon site of $tRNA^{Met}$ (Muramatsu *et al.*, 1988; Pallanck and Schulman, 1991; Schulman and Pelka, 1988).

Although the complex structure between *E. coli* methionyl-tRNA synthetase and $tRNA^{Met}$ has not been solved yet, the available biochemical and biophysical data suggest that the C-terminal domain should be responsible for the interaction with the anticodon part of $tRNA^{Met}$ (Meinnel *et al.*, 1991; Valenzuela and Schulman, 1986). Mutation experiments showed that W461 is the critical amino acid for the binding to the anticodon of $tRNA^{Met}$ and the peptide region around W461 is not involved in other activities of the enzyme, such as the binding to methionine and ATP (Kim and Schimmel, 1992). Several other residues neighboring W461 also seem to play a role in tRNA binding (Kim *et al.*, 1993; Kim *et al.*, 1994). It has been proposed that this tryptophane may make a base-specific hydrogen bond with the C of the CAU anticodon of $tRNA^{Met}$ (Schulman and Pelka, 1990).

To understand the molecular mechanism for the interaction with the anticodon of $tRNA^{Met}$, W461 of *E. coli* methionyl-tRNA synthetase was subjected to saturation mutagenesis. A total of 12 substitution mutants were obtained from this mutagenesis. The substituents include acidic (glutamic acid), basic (lysine and arginine), aliphatic (valine, leucine) and polar (serine and asparagine) and aromatic (histidine, tyrosine, phenylalanine) amino acids. In addition, substitutions for alanine and cysteine were also found.

The effect of mutation on the protein structure was tested by immunoblotting of *E. coli* methionyl-tRNA synthetase mutants. If a protein does not have a stable conformation due to a mutation, it would be susceptible to proteolytic attack and thus would be degraded away. Immunoblotting with rabbit antibody raised against the *E. coli* methionyl-tRNA synthetase was used to monitor the *in vivo* stability of the protein. Regardless of the substituted residues, the protein showed the same degree of stability as the wild-type enzyme (Table 1). This result suggests that the mutations occurring at the position 461 did not affect the protein structure. The result is also consistent with the fact that W461 is exposed to the surface of the protein and is not involved in maintaining the protein structure. Also, molecular dynamics simulation suggested that the loop region including W461 is the most flexible part of the structure with a movement of more than 4 Å rms (Ghosh *et al.*, 1991). Therefore, any structural deformation induced by the amino acid substitutions is buffered by this flexibility and does not propagate to disturb the whole protein structure.

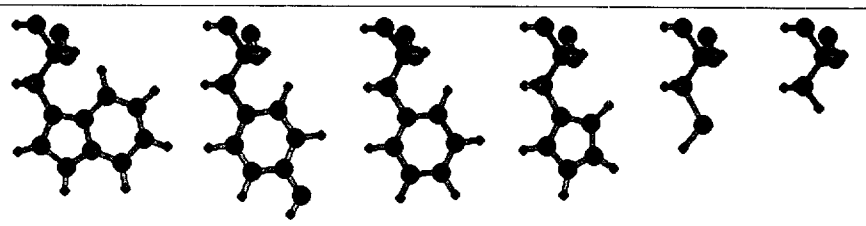
Table 1. *In vivo* stability and activity of *E. coli* methionyl-tRNA synthetase and substitution mutants at the position 461

Mutants	Stability	Complementation
W461 (Wild-Type)	+	+
W461Y	+	+
W461F	+	+
W461H	+	+
W461C	+	+
W461A	+	+/−
W461S	+	−
W461V	+	−
W461L	+	−
W461E	+	−
W461N	+	−
W461K	+	−
W461R	+	−

Note : The protein stability was determined by immunoblotting of cell extracts expressing each mutant using rabbit antibody raised against *E. coli* methionyl-tRNA synthetase. The mark + indicates that the stable protein conformation is made. The genetic complementation test was carried out to determine the *in vivo* enzyme activity of the mutants in the *E. coli* strain lacking the functional methionyl-tRNA synthetase. The mark + represents that the protein supports the cell growth at 42℃. The mark +/− represents that the colony was detected after 2 day incubation (see Materials and Methods for detail).

The *in vivo* activity of all the mutants was tested for their ability to complement the *E. coli* strain in which a gene encoding methionyl-tRNA synthetase (*MetG*) was ablated. The mutants with substitutions of tyrosine, phenylalanine, histidine and cysteine complemented the *MetG* null strain whereas others did not (Table 1). The cells expressing W461A mutant showed tiny colonies after 2 day incubation, which can be taken to indicate marginal activity to maintain cell viability. The active substitutions mainly consist of aromatic residues (Table 1). Considering that the wild-type residue is tryptophane, the aromatic ring structure seems to be an important determinant for the anticodon binding.

To compare the aminoacylation activity of the mutant with the wild-type enzyme, cell extracts from each mutant enzyme were prepared and *in vitro* aminoacylation assay was conducted. The activity showed a gradual decrease from the wild-type to the mutants following the order of tyrosine, phenylalanine, histidine, cysteine and alanine substitution (Fig. 2). The order of these enzyme activities fits well with that of the Van der Waals volume of each amino acid. This result implies that the size of the side-chain is another important



Amino acid at Position 461	Trp	Tyr	Phe	His	Cys	Ala
Van der Waals Volume ($Å^3$)	163	141	135	118	86	67
Relative Aminoacylation Activity (%)	100	38	19	12	8	4

Fig. 2. The side-chain structures of the wild-type (tryptophane) and substituted amino acids at the position 461 of *E. coli* methionyl-tRNA synthetase. The Van der Waals volumes (Darby and Creighton, 1993) and the relative aminoacylation activities of the enzymes containing each amino acid were shown.

factor for the interaction with the anticodon. It might be that the binding pocket made by the anticodon site of tRNA is well packed by W461 of *E. coli* methionyl-tRNA synthetase in the complex, and the amino acids with smaller side-chains than tryptophane are energetically less favored for the binding to the anticodon.

It is also interesting to note that the W461C mutant complemented the *E. coli* tester strain whereas the W461S mutant did not. The chemical difference between the two residues is that cysteine has sulfur (Van der Waals radius=1.85 Å) connected to C_β whereas serine has oxygen (Van der Waals radius=1.40 Å at the same place (Mijima *et al.*, 1987). Due to this difference, cysteine is more hydrophobic and bigger than serine, which could make cysteine more favored for the interaction with the anticodon of $tRNA^{Met}$. Perhaps, the space of the anticodon can accomodate the cysteine residues but is not large enough to accomodate the non-complementing aliphatic residues. The aminoacylation activity of the W461Y mutant is almost two fold higher than that of the mutant W461F, although the size of tyrosine is slightly bigger than that of phenylalanine (Fig. 2). This result implies that the hydrogen bonding potential of tyrosine at the aromatic hydroxyl group may be important for the interaction with the anticodon. In summary, the results obtained from *in vivo* genetic complementation and *in vitro* aminoacylation assay suggest that the aromatic ring structure, Van der Waals volume and hydrogen bonding potential of side-chains are important factors for productive interaction with the anticodon of $tRNA^{Met}$. We do not know how all these factors are combined to interact productively with the anticodon of $tRNA^{Met}$. More accurate interpretation of the results would be possible when structural information on the complex between *E. coli* methionyl-tRNA synthetase and $tRNA^{Met}$ becomes available.

Acknowledgement

This work was supported in part by the Basic Science Research Institute Program (Project No. BSRI-94-4417) of Ministry of Education.

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