Volume 289, number 2, 217–220 FEBS 10141 September 1991

ADONIS 001457939100853Y

Identification of potential amino acid residues supporting anticodon recognition in yeast methionyl-tRNA synthetase

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Received 21 May 1991; revised version received 8 July 1991

Sequence comparisons among methionyl-tRNA synthetases from different organisms reveal only one block of homology beyond the last β strand of the mononucleotide fold. We have introduced a series of semi-conservative amino acid replacements in the conserved motif of yeast methionyl-tRNA synthetase. The results indicate that replacements of two polar residues (Asn^{584} and Arg^{588}) affected specifically the aminoacylation reaction. The location of these residues in the tertiary structure of the enzyme is compatible with a direct interaction of the amino acid side-chains with the tRNA anticodon.

Methionyl-tRNA synthetase; Site-directed mutagenesis; Anticodon binding region

1. INTRODUCTION

The structure of a fully active fragment of the *E. coli* methionyl-tRNA synthetase (MetRS) complexed with ATP has recently been resolved at high resolution [1], but information about the regions that participate in the complex with the tRNA has only been generated by genetic and chemical cross-linking experiments. The terminal adenosine of $tRNA^{Met}$ is proximal to residues Lys^{61} and Lys^{335} when bound to the synthetase [2] and peptide region 528–533 has been proposed to play a role in guiding the acceptor stem in the active site [3]. The interaction with the anticodon bases which specify methionine acceptance in the tRNA [4] is restricted to residues 451–467 [5–7]. This region is characterised by the presence of a tryptophane residue (Trp^{461}) which is conserved in all methionyl-tRNA synthetases with the exception of the yeast cytoplasmic enzyme, and has been shown to interact positively with methionine anticodons and negatively with non-methionine anticodons [6,7]. Recently, a structural model of the *E. coli* $tRNA^{Met}$–MetRS complex has been suggested based on structural similarities between methionyl- and the tRNA bound *E. coli* glutaminyl-tRNA synthetase [8]. In this model, the positioning of the anticodon near Trp^{461} at the extreme periphery of the molecule is determined by a long α-helix which extends along the entire length of the D and anticodon stems. In a previous attempt to identify the hydrogen bond network between amino acid residues of yeast methionyl-tRNA synthetase and the anticodon triplet of the cognate $tRNA^{Met}$, we have analysed an α-helical region that corresponded to the *E. coli* methionyl-tRNA synthetase anticodon binding region (helix H4′ in Fig. 1). We have mutated more than 10 potential amino acid donors in the equivalent yeast sequence and found that these mutations had little effect on tRNA recognition (unpublished results). This result suggests that the determinants of the anticodon binding region in the yeast system are located elsewhere in the molecule. New contacts could be identified by modelling the interaction of the tRNA with a structural model of the yeast methionyl-tRNA synthetase. In particular we identified an asparagine residue that is directly adjacent to the anticodon when the tRNA is docked to the synthetase. By sequence comparison, we noted that this asparagine residue falls in a highly conserved amino acid region that is found in all methionyl-tRNA synthetases so far sequenced. Therefore we mutated this cluster of conserved residues and analysed their effect in tRNA aminoacylation.

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2. MATERIALS AND METHODS

2.1. *Strains and plasmids*

The *E. coli* strain JM101 [6] was used as recipient for the various plasmids and M13 phages. The *E. coli* strain TG1 was used in transformations by phosphorothioate DNA. The genotype of TG1 is indicated by the supplier (Amersham). Plasmid pMVT was the source of isolation of MES1 gene. It allows overexpression of the MES1 protein in a recipient strain FF1 (*mes1,ura3*) that harbours a chromosomally altered methionyl-tRNA synthetase whose activity is undetectable in vitro [9].

2.2. *Enzymes and reagents*

Restriction endonucleases, T4 DNA ligase and *E. coli* DNA polymerase I (Klenow fragment) were purchased from Boehringer Mann-

Fig. 1. Juxtaposition of the yeast MetRS model (catalytic core) with the phosphate backbone $tRNA_i^{Met}$. The phosphate backbone of the tRNA and the α-carbon backbone of the protein starting from residue 192 are shown. Region A is drawn in heavy lines and represents the HIGH and the KMSKS regions. Arrows are pointing to the positions of the carbon α spheres of the residues mentioned in the text. The coordinates of S1', H4' and H1' are taken from Brunie et al. [1].

heim (Germany). [α-^{35}S]dATP and ^{125}I-protein A were purchased from Amersham (UK).

2.3. *Site-directed mutagenesis of the MES1 structural gene*

The Amersham mutagenesis system based on the method of Eckstein et al. [10] was used to create single base pair mutations. The *Hin*dIII–*Bam*HI fragment from the MES1 gene was subcloned into M13mp19 and used as DNA template. After transformation of the TG1 strain, positive clones were identified by sequencing the DNA of four AmpR colonies using the Sanger dideoxy method [11].

2.4. *Reconstruction of mes1 mutants*

The DNA (1 μg) from M13 phages carrying the mutated MES1 sequences were digested by *Cla*I and *Hin*dIII restriction enzymes (2 units) and the resulting digest was ligated directly into plasmid pMVT that has been successively digested with *Tth*III, *Cla*I, *Hin*dIII (partial digest) and dephosphorylated. We transformed the FF1 recipient with the ligation mixture and selected colonies with the Ura3$^+$ phenotype in the presence of methionine (100 μg/ml). Positive clones were counter-selected for the Met$^-$ phenotype. Both activities, the ATP–PPi exchange and the tRNA aminoacylation were measured for one Met$^+$ colony and each Met$^-$ colony. The complete sequence of the reconstructed gene was verified. We did not find additional mutations to those introduced by site-directed mutagenesis.

2.5. *Methionyl-tRNA synthetase activity measurements in crude extracts*

Cytoplasmic methionyl-tRNA synthetase was tested in crude extracts from exponentially growing cells, obtained by mechanical breakage using glass beads. Aminoacylation reactions were carried out at 25°C under the following conditions: 144 mM Tris-HCl, pH 7.8; 5 mM dithiothreitol; 2 mM ATP; 10 mM $MgCl_2$; 0.1 mM [^{14}C]methionine (51 μCi/μmol); 6 mg/ml yeast tRNA (the fraction of $tRNA^{Met}$ is 2%); and 5 mg/ml bovine serum albumin. At various time intervals 40 μl aliquots from a 200 μl reaction mixture were spotted onto Whatman paper disks and quenched by 5% trichloroacetic acid. Radioactivity was counted in a toluene-based scintillant. The ATP–pyrophosphate exchange assays were done at 25°C using: 144 mM Tris-HCl, pH 7.8; 2 mM ATP; 2 mM [^{32}P]PPi (0.35 μCi/μmol); 5 mM methionine; 10 mM potassium fluoride (an inhibitor of endogenous pyrophosphatase); 0.1 mM phenylmethanesulphonylfluoride, and various amounts of crude enzyme. Labeled ATP was adsorbed onto charcoal, washed with water and the radioactivity was monitored by scintillation counting as above. One unit of activity corresponded to 1 nmol of [^{14}C]methionine incorporated into tRNA per min or 1 nmol of [^{32}P]phosphate incorporated into ATP per min. The specific amount of methionyl-tRNA synthetase in the protein sample was estimated by Western blotting. Protein samples were run on 10% polyacrylamide gels in the presence of 0.1% sodium dodecylsulphate. Conditions for the transfer of proteins to nitrocellulose membranes were as described in Schleicher and Schuell manual No2. The protein band corresponding to methionyl-tRNA synthetase was detected using DEAE-Sephadex-purified antibodies (100 μg) and ^{125}I-protein A (0.7 mCi at 50 mCi/mg).

3. RESULTS AND DISCUSSION

3.1. *Sequence homology between various methionyl-tRNA synthetases*

The overall homology between methionyl-tRNA synthetases from various sources [12–14] beyond the last β strand of the Rossmann fold is low (20% identities) compared to that of the nucleotide binding fold (40%). However, small regions of conserved amino acid residues could be identified (Fig. 2). One of these is the GNLV-R consensus sequence that is found in all methionyl-tRNA synthetases so far sequenced. A spectrum of semi-conservative amino acid replacements was sought for residues Asn^{584} and Arg^{588} of the yeast cytoplasmic enzyme. At position 584, we have analysed the steric effect of a methylene group and a negative charge on the asparagine side chain by producing the mutants Asn^{584}Gln and Asn^{584}Asp. To distinguish whether the side chain of arginine 588 makes a hydrogen or an ionic bond with the tRNA we have tested the replacements

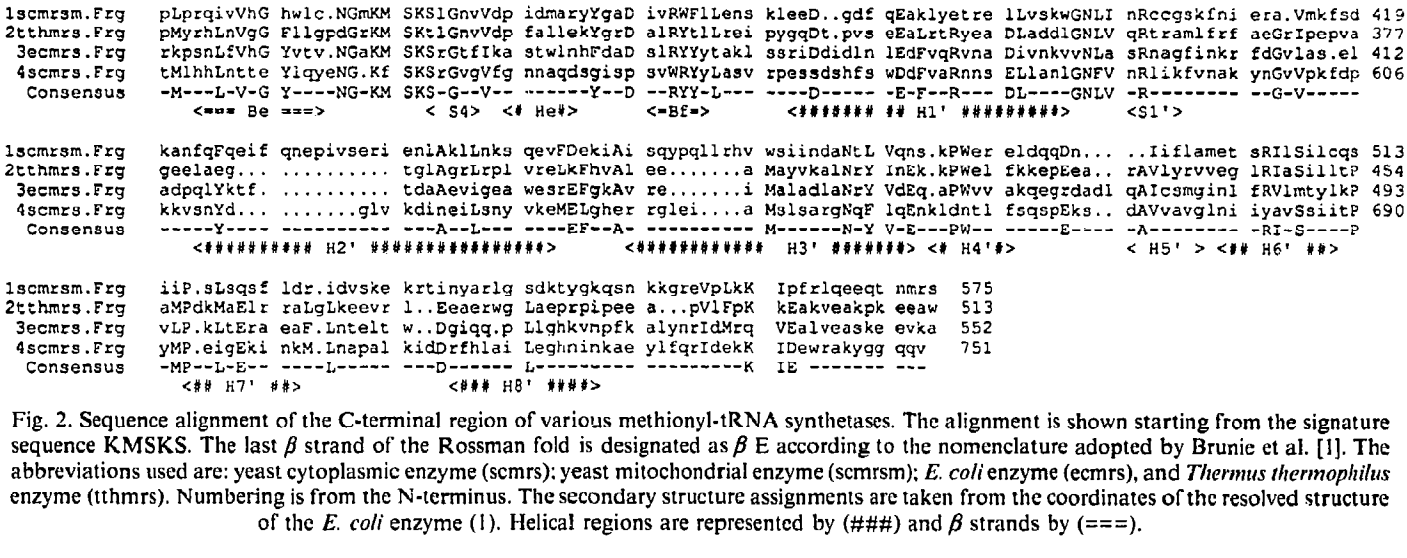

Fig. 2. Sequence alignment of the C-terminal region of various methionyl-tRNA synthetases. The alignment is shown starting from the signature sequence KMSKS. The last β strand of the Rossman fold is designated as β E according to the nomenclature adopted by Brunie et al. [1]. The abbreviations used are: yeast cytoplasmic enzyme (scmrs); yeast mitochondrial enzyme (scmrsm); *E. coli* enzyme (ecmrs), and *Thermus thermophilus* enzyme (tthmrs). Numbering is from the N-terminus. The secondary structure assignments are taken from the coordinates of the resolved structure of the *E. coli* enzyme (1). Helical regions are represented by (###) and β strands by (===).

of Arg into glutamine and lysine. To test the importance of aromatic hydrophobic interactions we have changed Phe[585] into alanine.

3.2. *Rationale for the selection of mutant enzymes and measurement of the enzyme activities*

Because it was very difficult to obtain mutated DNA by the phosphorothioate method using the phagemid pMVT (containing the entire MES1 gene), we performed the mutagenesis in the MES1 *Hin*dIII–*Bam*HI fragment that covers most of the adenylate binding domain and the entire C-terminal helical domain. The *mes*1 mutants were reconstructed directly from a ligation mixture of two independent *Hin*dIII–*Cla*I digests of pMVT vector and M13 recombinants bearing the mutated mes1 sequences. As we used a ligation mixture to transform the recipient strain having a defective methionyl-tRNA synthetase, 4 possibilities can be envisaged: complementation by the pMVT vector due to incomplete digestion and/or dephosphorylation; complementation by the mutant methionyl-tRNA synthetase whose in vivo aminoacylation level is high enough to sustain growth, failure of complementation due to size trimming of the 5′ end overhangs or ligation of concatemers, or finally, failure of complementation due to non functional methionyl-tRNA synthetase mutants. The latter possibility can be distinguished from all the other ones by determining the methionine phenotype and measuring the enzyme activities of the corresponding colonies. Phenotypically, they should be Met$^-$ and the mutant protein should display impaired ATP–PPi exchange activity but hampered tRNA aminoacylation if the mutation specifically affects the tRNA-binding or the transfer step of the activated amino acid. Mutants with only partially altered $tRNA^{Met}$ aminoacylation activity give rise to colonies with the Met$^+$ phenotype. These mutants will be distinguished from wild type enzyme on the basis of the activity measurements. The strategy that led to the isolation of the desired mutants involved the following steps: (i) transformation of the recipient in the presence of methionine; (ii) check of the methionine phenotype of the Ura3$^+$ transformants, and (iii) measurements of both activities in a Met$^+$ and a Met$^-$ colony. The results are shown in Table I. Methionyl-tRNA synthetase mutants could be isolated from Met$^-$ colonies for N584Q, N584D, N584A, R588Q and R588K. Mutant F585A was not obtained by this genetic selection procedure. Our analysis did also select for second site revertants of the mes1 locus. These clones grew well in the absence of methionine but showed little ATP–PPi exchange activity and tRNA aminoacylation (20 times less than the overproduced ATP–PPi exchange activity) but were not further analysed. In order to prove that the cloned mes1 mutants express a full-length protein we performed a SDS-polyacrylamide gel-electrophoresis. Fig. 3 shows that this is indeed the case for all mutants analyzed and we could estimate the specific level of each mutant by immuno-blot analysis using polyclonal methionyl-tRNA synthetase antibodies. The specific activity of mutants N584D and R588Q in the crude extracts is 0.5–1.5% of that of the wild type and undetectable for mutants R588K, N584Q and N584A (Table I). These effects are spectacular considering the

Table I

Measurement of the specific activity of wild type and methionyl-tRNA synthetase mutants

MetRS	In vivo complementation	Relative specific activity (%)	
		ATP/PPi exchange	Aminoacylation
wild type	+	100	100
N584D	–	60	0.5–1.5
N584Q	–	65	0.3
R588A	–	65	0
R588K	–	50	0.3
R588Q	–	75	0.5–1.5

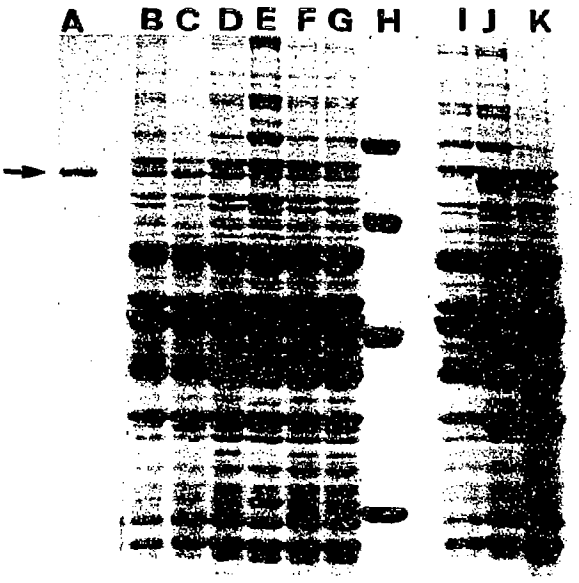

Fig. 3. SDS-polyacrylamide gel analysis of yeast methionyl-tRNA synthetase (MetRS) mutants. Crude protein extracts from mutants (30 μg) and wild type enzyme (30 and 60 μg) were separated on a 10% SDS-polyacrylamide gel. (A) 0.5 μg of purified MetRS; (B) N584A; (C) N584D; (D) N584Q; (E) R588A; (F) R588K; (G) R588Q; (H) protein marker; (I) recipient strain FF1; (J) and (K) 60 and 30 μg of wild type MetRS. The arrow indicates the position of the native MetRS.

fact that in most cases semi-conservative amino acid substitutions were made. All these residues belong to the end of an α-helix that is directly adjacent to the CAU anticodon of the tRNA according to our docking experiments and may, therefore, represent residues that are implicated in tRNA binding or in the catalytic step. To clarify this problem, measurement of K_m and k_{cat} parameters of the purified proteins will be necessary. We have also tested the potential of the respective yeast mutants to aminoacylate $tRNA^{Met}$ transcripts with altered anticodon specificities (GAC, CCU, UAU and CAG). No mis-aminoacylation could be measured in vitro with the protein crude extracts previously purified by DEAE-cellulose chromatography. This result corroborates the absence of lethality noted when the corresponding yeast transformants were grown in the presence of methionine.

Acknowledgement: We thank G. Nussbaum for skilful technical assistance.

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