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Structure – function relationship of arginyl-tRNA synthetase from *Escherichia coli*: isolation and characterization of the *argS* mutation MA5002

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ABSTRACT

The *Escherichia coli* K12 *argS* MA5002 mutant appears to have a functionally altered arginyl-tRNA synthetase (ArgRS). The gene coding for this enzyme was isolated from *E.coli* genomic DNA using the PCR procedure and inserted into a pUC18 multicopy vector. Sequencing revealed that it differs from the wildtype ArgRS structural gene only by one mutation: a replacement of a C by an A residue which results in substitution of an arginine by a serine at position 134, located two residues downstream from the HVGH consensus sequence. As compared to the genomic enzyme level, this recombinant vector, containing the mutated gene, produces in *E.coli* JM103, about 100 times as much modified ArgRS. This enzyme was obtained nearly pure after only two chromatographic steps; it exhibits a 4 – 6 times as low activity and a 5 times as high Km value for ATP as the wildtype enzyme in the aminoacylation and ATP-PPi reactions; Km values for arginine and $tRNA^{Arg}$ remained unaltered. The position of this mutation and its effect on enzymatic properties suggest the implication of arginine 134 in ATP binding as well as in the activation catalytic process.

INTRODUCTION

Arginyl-tRNA synthetase like glutamyl- and glutaminyl-tRNA synthetases differ from the other aminoacyl-tRNA synthetases in that they require their cognate tRNA to catalyse the ATP-PPi exchange reaction (1,2). In spite of extensive investigations performed by several groups, no definitive answer could be brought so far in favour of one of the two most frequently proposed mechanisms accounting for this peculiar enzymatic property: either a two-step mechanism, where tRNA binding is a prerequisite for adenylate formation (1), or ATP, arginine and tRNA coreact in a concerted way leading to a one step aminoacylation of tRNA (3). However some thorough kinetic investigations (4), such as studies (5) showing that *E.coli* ArgRS catalyses the ATP-PPi exchange in the presence of non chargeable $tRNA^{Arg}$ having a 3′ terminal deoxyadenosine, support the idea of a classical two-step mechanism.

Recently we isolated and sequenced the structural gene coding for *E.coli* arginyl-tRNA synthetase together with its 247 bp and 399 bp 5′ and 3′ flanking regions (6). Its amino acid sequence exhibits no relevant similarities with those of other aminoacyl-tRNA synthetases except for the presence of an HVGH sequence homologous to HIGH. This consensus motif, initially identified by Webster *et al.* (7), represents one of the most significant homologies observed up to now between aminoacyl-tRNA synthetases. In the case of TyrRS from *B.stearothermophilus* it was demonstrated that its two flanking histidine residues are implicated in ATP binding (8,9).

In order to investigate the structure-function relationship in *E.coli* ArgRS, we first tried to characterize the mutation described by Cooper *et al.* (10). The corresponding mutant (MA 5002) which was isolated on the basis of its resistance to canavanine, an inhibitory analogue of arginine , grows more slowly than the wildtype strain in the absence or presence of arginine and exhibits reduced arginyl-tRNA synthetase activity. To determine the enzyme mutation(s) responsible for these modifications, we first isolated the mutated *argS* gene out of the genomic DNA by the PCR procedure. The synthesised DNA fragment was inserted into a pUC18 vector and thorougly sequenced to localize possible mutations. The transformant strain containing this recombinant plasmid overproduces mutant ArgRS more than 100 fold as compared to the wildtype genomic enzyme. This allowed an efficient purification in a few steps of high amounts of the mutant enzyme which was analysed for its catalytic parameters.

MATERIALS

Nucleotides, oligonucleotides and DNA

Unfractionated *E.coli* tRNA was purchased from Boerhinger Mannheim. 2′ deoxy- and 2′3′ dideoxynucleoside 5′-triphosphates were from Pharmacia. [α-^{35}S]-dATP (410 Ci/mmole) was from Amersham. Oligonucleotide primers for sequencing and chain reaction polymerisation were synthesized on an Applied Biosystems 381A DNA apparatus.

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CsCl purified double stranded plasmidic DNA was prepared by the alkaline method according to Maniatis *et al.*(11). Rapid small-scale plasmidic DNA preparation were carried out by the method of Birnboim and Doly (12). If not specified, DNA manipulations were performed as described (11).

Chemicals and enzymes

Taq DNA polymerase from *Thermus aquaticus* was obtained from Bioexcellence and Beckman. Modified T7 DNA polymerase was from Pharmacia, T4 DNA ligase from Boehringer-Mannheim, and bacterial alkaline phosphatase from Appligene. [^{14}C]L-arginine was purchased from the CEA (Saclay, France), HA Ultrogel was from LKB-IBF and DEAE Sephacel from Pharmacia.

EXPERIMENTAL PROCEDURES AND RESULTS

Transformation and analytical procedures

Transformation of *E.coli*, agarose and denaturing polyacrylamide gel electrophoresis, recovery of DNA fragments from agarose were carried out by standard procedures (11).

Isolation of the mutated ArgRS gene by enzymatic amplification

Genomic DNA of the *E.coli* K12 mutant MA5002 was prepared according to (13). Synthesis of a 1.9 kbp DNA fragment containing the gene of ArgRS was conducted directly from its 3′ and 5′ ends by the PCR procedure in the presence of the appropriate oligonucleotide primers: the reaction mixture (100 µl) present in a microcentrifuge tube contained 1 µg genomic DNA, 55 mM Tris-HCl pH 8.8 (at 42°C), 16 mM ammonium sulphate, 5 mM $MgCl_2$, 50 mM KCl, 1 mM of each deoxynucleoside triphosphate, 2 units of Taq DNA polymerase and 1 µM of primers complementary to each end of the ArgRS gene and bordered by a *Hin*dIII sequence (Figure 1). The mixture was overlaid with 200 µl of mineral oil (Merck) to prevent evaporation and transferred to a programmable heat block (Techne Programmable Dry Block PHC-1). After heating for 4 min at 92°C to denature the genomic DNA, the first primer annealing step was achieved at 35°C for 2 min to optimize the number of hybridized molecules, increasing however the probability of non-specific hybrid formation. The bound primers were finally extended for 5 minutes at 70°C. The —denaturation, annealing, extension— cycle was repeated 25 times under the same conditions except that denaturations were performed for 2 min and hybridizations for 2 min at 62°C: this much more stringent temperature (to reduce non specific annealing) was estimated according to the Wallace rule (14) taking into account the nucleotide composition of the primers. Analysis of a 10 µl aliquot of the final polymerization mix by agarose gel electrophoresis (Figure 2) revealed a band whose length (1.9 kbp) corresponds to that of the expected ArgRS gene. As could be estimated from the band intensity, more than 1 µg of this specific fragment was synthesized starting from 1 µg genomic DNA.

Cloning and sequencing of the amplified fragment

To clone the mutated *argS*, the amplified 1.9 kbp fragment was digested by *Hin*dIII, purified from PCR components by electrophoresis on an agarose gel and inserted into the corresponding site of the dephosphorylated pUC18 vector.

Sequencing of the double stranded plasmidic DNA was achieved by the dideoxy chain termination method in the presence of modified T7 DNA polymerase (15). The whole primary structures of two cloned inserts coming from different transformant clones were determined in order to detect among the mutations those possibly generated during the PCR step. From sequence comparison it appears that the *argS* gene from mutant

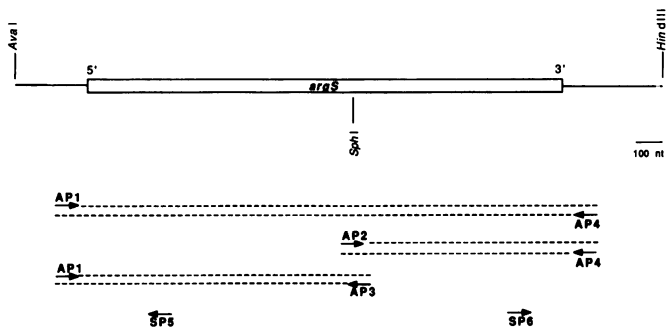

Figure 1: Amplification and sequencing strategy of the *argS* region.
AP1: CGCAAGCTTTACGCCACGCGCACGTTGCTGACG;
AP2: CACTGCATGCCGATCGCGTGCTGTATTACATCG;
AP3: ATGCCTGCATCAGGTGTTGATGCTGACGG;
AP4: CGCAAGCTTCTAACCCTGATCAGGCTTCACTCTCACG;
SP5: GCACCTGCTCTGCTAATTGTCGCGGTG;
SP6: ACGATCTGGCCGGTCTGTTC.
Arrows indicate the 5′ → 3′ orientation of the primers. Primers AP1 and AP4 hybridize to positions −112 to −89 and 1744 to 1772 respectively with respect to the first nucleotide of the coding region (6); underlined nucleotides are non complementary to the wildtype *argS* sequence and correspond to the recognition site for the *Hin*dIII restriction enzyme. AP2 and AP3 are primers that hybridize in the central part of the gene, from 980 to 1012 and 1017 and 1045 respectively. SP5 and SP6 are sequencing primers complementary to positions 160−187 and 1571−1591 respectively. nt, nucleotides.

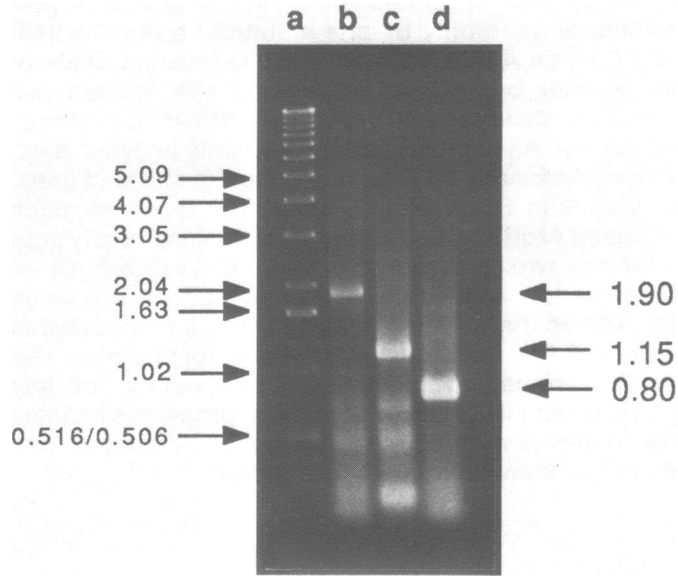

Figure 2: Agarose gel electrophoresis of PCR extension products. PCR amplifications were performed using the oligonucleotide primers described in Fig.1. One tenth of the final polymerization mixtures was fractionated on a 1% agarose gel. Numbers refer to sizes in kbp. Lane a, DNA ladder. Lane b, amplification of the 1.9 kbp was achieved using primers AP1 and AP4 which create *Hin*dIII sites at both ends (to clone into pUC18 vector). Lanes c and d, the amplification of the gene was also performed using primers AP1−4 to produce two DNA fragments of 0.8 and 1.15 kbp, overlapping each other on a 50 bp central region which contains a unique *Sph*I restriction site.

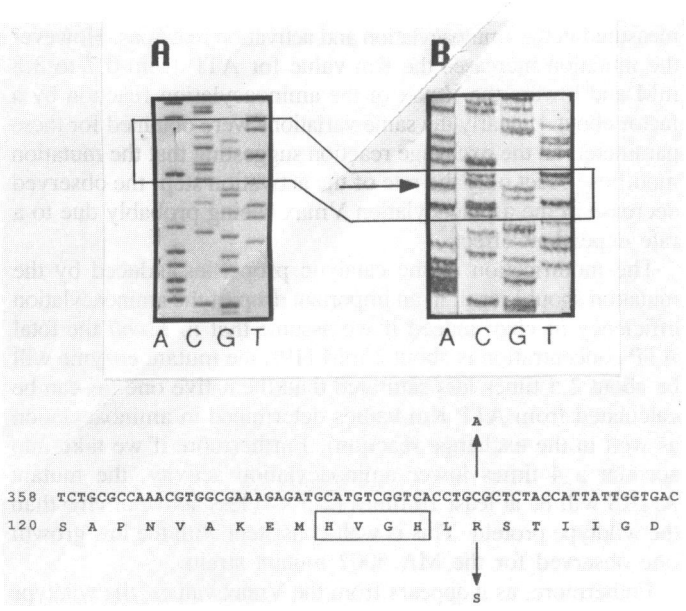

Figure 3: Sequences showing the location of the mutation in ArgRS from strain MA5002. The entire 1.9 kbp *Hin*dIII fragment was sequenced using primers 1–6. The autoradiographs shows sequences of *argS* from strain MA5002 (B) and wildtype (A) in the region of the mutation. The position of the mutation is indicated by an arrow. Below, the mutation is indicated on the mRNA identical DNA strand and results in a Arg (C) to Ser (A) change at codon 134.

MA5002 differs from the wildtype gene only by one mutation: a C to A transition in the codon 134 leading to the substitution of an arginine by a serine (Figure 3).

Purification of the mutated arginyl-tRNA synthetase

About 10% of the crude extract protein prepared from *E.coli* JM103 carrying pUC18-1.9 kbp is mutant ArgRS as determined by immunological titration (16). This more than 100 fold overproduction compared to the wildtype strain favoured efficient purification of the plasmidic ArgRS in a few chromatographic steps: the transformant JM103 pUC18-1.9 kbp was grown at 37°C to stationary phase in 6000 ml LB containing 200 μg per ml ampicillin. Cells were harvested by centrifugation, washed with 500 ml of 10 mM Tris-HCl pH7.5, 1 mM EDTA, resuspended in 60 ml of 100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA and finally lysed (20–30 g wet cell paste) by 6 times sonication for 20 sec at 100 V with an Ultrasons-Annemasse apparatus (Type 250TS20K). The lysate was cleared by centrifugation successively at 6000×g for 30 min and 105,000×g for 2 hours. Protein concentration was determined as described (17). The resulting supernatant was loaded onto a DEAE Sephacel column (id = 5.5 cm, h = 12.5 cm) equilibrated in 20 mM potassium phosphate buffer pH 7.5. The column was washed with 250 ml of the same buffer and developed with 1500 ml of potassium phosphate pH 7.5 (20 mM to 500 mM) at a flow rate of 120 ml/hour. Active fractions were pooled, dialysed extensively against 10 mM potassium phosphate buffer pH 7.5 and applied to a HA Ultrogel column (id = 2.5 cm, h = 50 cm) equilibrated with the same buffer; then the column was washed with 200 ml of this last buffer and eluted with 1000 ml of a linear gradient of potassium phosphate buffer pH 7.5 going from 10 mM to 50 mM at a flow rate of 60 ml/hour. After this step, the enzyme (27 mg) was about 95% pure as estimated from SDS-PAGE (Figure 4).

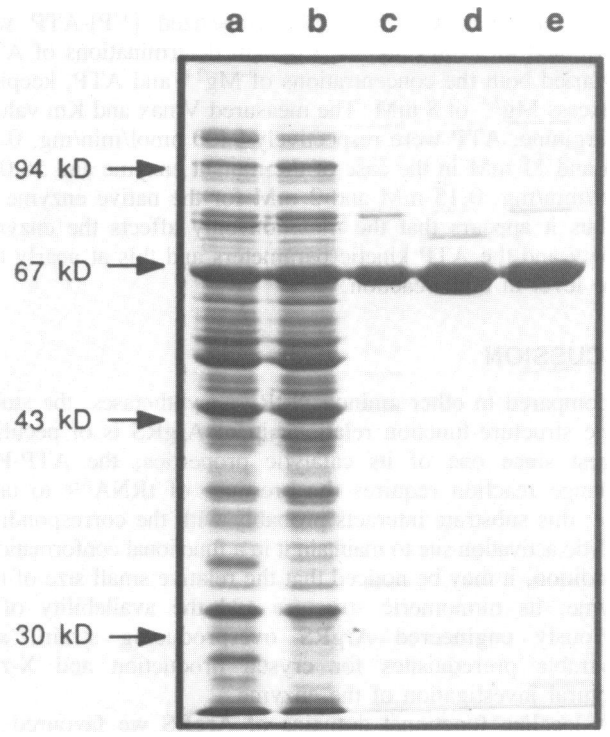

Figure 4: Purification of mutant arginyl-tRNA synthetase from the strain MA5002. SDS PAGE was carried out according to 23. After electrophoresis, the gel was stained by Coomassie Blue. Lane a, 30 μg of crude extract from strain MA 5002. Lane b, 30 μg of protein from the 105,000×g supernatant. Lane c, 3 μg of ArgRS from the DEAE Sephacel fraction. Lane d, 6 μg of ArgRS from the HA Ultrogel. Lane e, 4 μg of the wild type ArgRS prepared as in (6).

Measurements of kinetic parameters

The specific activities of the enzymes in aminoacylation or ATP-PPi exchange were measured either directly with crude extracts or with partially purified or pure proteins.

Aminoacylation reaction: A reaction mixture contained 50 mM Tris-HCl pH7.5, 30 mM KCl, 20 mM $MgCl_2$, 0,1 mM [^{14}-C]L-arginine (45,000 cpm/nmole), 10 mM ATP, 6 mg/ml unfractionated *E.coli* tRNA (it contains about 2% $tRNA^{Arg}$), 5 mM dithio-erythritol, 0.1 mg/ml bovine serum albumin and appropriate amounts of enzyme. After various incubation times at 37°C, [^{14}C]-arginyl-$tRNA^{Arg}$ was assayed in 40 μl of the reaction mixture by the method described previously (18) and initial rates were determined. One activity unit is defined as the amount (in mg) of enzyme which aminoacylates one nanomole of $tRNA^{Arg}$ in one minute under the above conditions. The measured Vmax and Km values for arginine, ATP and $tRNA^{Arg}$ were respectively 3,500 nmol/min/mg, 11 μM, 4.3 mM and 4.2 μM for the mutant enzyme and 14,000 nmol/min/mg, 9 μM, 0.7 mM and 3 μM for the native enzyme; these parameters were determined under the conditions described above except that the excess of Mg^{2+} over ATP was maintained constant at 10 mM in the case of Km measurements for ATP.

ATP-PPi exchange reaction: The standard reaction mixture contained 100 mM sodium Hepes pH 7.2, 10 mM $MgCl_2$, 2 mM ATP, 2 mM [^{32}P]-PPi (1–2 cpm/pmole), 5 mM L-arginine, 10 mM KF (to inhibit pyrophosphatase activity), 12 mg/ml unfractionated *E.coli* tRNA and enzyme. After various

incubation times at 37°C, the synthesized [^{32}P]-ATP was determined in 50 µl aliquots. For Km determinations of ATP we varied both the concentrations of Mg^{2+} and ATP, keeping an excess Mg^{2+} of 8 mM. The measured Vmax and Km values for arginine, ATP were respectively 9400 nmol/min/mg, 0.11 mM and 11 mM in the case of the mutant enzyme and 56,000 nmol/min/mg, 0.15 mM and 2 mM for the native enzyme.

Thus it appears that the mutation only affects the enzyme activity and the ATP kinetic parameters and this at nearly the same level in both reaction.

DISCUSSION

As compared to other aminoacyl-tRNA synthetases, the study of the structure-function relationship of ArgRS is of peculiar interest since one of its catalytic properties, the ATP-PPi exchange reaction requires the presence of $tRNA^{Arg}$ to take place; this substrate interacts probably with the corresponding catalytic activation site to maintain it in a functional conformation. In addition, it may be noticed that the relative small size of the enzyme, its monomeric structure and the availability of a previously engineered ArgRS overproducing strain, are favourable prerequisites for crystal production and X-ray structural investigation of the enzyme.

To localize functional domains of ArgRS we favoured an approach which consists in measuring the effect induced by polypeptide chain modifications on the catalytic parameters. This led us to first analyse a mutant described earlier by Cooper *et al.* (10) which, as they showed, exhibits modified enzymatic properties relative to ArgRS.

The gene coding for the mutant enzyme was isolated from genomic *E.coli* DNA by the PCR method as a 1.9 kbp fragment that was inserted into a pUC18 plasmid. This *in vitro* technique for primer directed enzymatic amplification of specific DNA sequences is capable of synthesizing over a million copies of a specific target DNA sequence. It represents a form of *in vitro* molecular cloning that can accomplish, in an automated 3-step reaction, what otherwise takes days or weeks of biological growth, screening and biochemical purification. However it has to be noticed that in order to successfully apply this method, special attention has to be paid to the choice of the primer-template hybridization temperature. As was the case in our study, the determination of the optimal value of this critical parameter often requires several preliminary tests.

The recombinant pUC18 plasmid containing the mutant ArgRS gene expressed this enzyme at a level corresponding to about 10% of the total amount of the crude extract proteins. This favoured rapid isolation of the enzyme that was nearly pure after two chromatographic steps. Sequencing of the whole double stranded mutant structural gene of ArgRS revealed that a unique mutation was present which consisted in substitution of an arginine at position 134 by a serine. It appears that this mutation is located two residues downstream from a HIGH consensus box motif which, as demonstrated in the case of TyrRS from *B.stearothermophilus*, is implicated by both its histidine residues respectively in ATP binding and stabilization of the adenylate transition complex (8, 9). Although this consensus sequence is located in a much more central position (129−132) in ArgRS as compared to the other aminoacyl-tRNA synthetases, its functional identity can be assumed by considering the effect of the mutation at the adjacent position 134 on enzymatic properties: as was shown from kinetic investigations, this modification didn't affect the Km values neither for arginine nor for the $tRNA^{Arg}$ measured in the aminoacylation and activation reactions. However the mutation increases the Km value for ATP from 0.7 to 3.8 mM and lowers the Vmax of the aminoacylation reaction by a factor about 4; nearly the same variations were obtained for these parameters in the exchange reaction suggesting that the mutation modifies in fact only the rate of the activation step, the observed decrease in the aminoacylation Vmax beeing probably due to a rate dependent effect.

The modification of the catalytic properties induced by the mutation should result in an important drop of the aminoacylation efficiency *in vivo*; indeed if we assume that in *E.coli* the total ATP concentration is about 2 mM (19), the mutant enzyme will be about 2.5 times less saturated than the native one (as can be calculated from ATP Km values determined in aminoacylation as well in the exchange reaction). Furthermore if we take into account a 4 times lower aminoacylation activity, the mutant ArgRS will be at least 10 times (2.5×4) less active *in vivo* than the wildtype protein. This is well consistent with the low growth rate observed for the MA 5002 mutant strain.

Futhermore, as it appears from the Vmax values, the wildtype ArgRS compared to the mutant enzyme exhibits lower activation energy in both reaction steps; this let us to conclude that the increment of ATP binding energy due to arginine 134 is realized in stabilization of the enzyme transition state rather than the enzyme ATP complex. In the activation as well as in the aminoacylation step a binding energy variation of about 2 kcal, which corresponds to that of a hydrogen bond, can be calculated according to the formula developed by Fersht (20) taking into account both the kcat and Km values for mutant and wildtype enzymes.

The question may be raised as to whether this higher binding energy, due to the presence of an arginine residue at position 134 near the HVGH motif, reflects the existence of a direct interaction of this amino acid with ATP, or is the consequence of an indirect effect which improves the efficiency of the ATP binding domain. If we consider the tertiary structure of the equivalent HIGH domain in TyrRS from *B.stearothermophilus* (21) and *E.coli* MetRS (22) we observe that the position corresponding to arginine 134 in these enzymes (2 residues downstream from the HIGH) is located in an α helix; this structural element connected with an adjacent β strand constitutes, as demonstrated (21), the seat of ATP binding and amino acid activation.

Provided, as it may be reasonably assumed, that the HVGH structural context of ArgRS is homologous to that of the previous enzymes, it is more than likely that arginine 134, due to the length of its side chain which confers him a wide potential interaction area, may interact with residues located in the catalytical activation site as well as the bound ATP substrate; in the case of MetRS we could show by modelling, using the FRODO programme, that an arginine located two residues away from the HIGH motif in MetRS from *E.coli*, has several possibilities to establish several hydrogen bonds with ATP. Studies concerning further investigations of this point and more generally the localization of other functional domains are now in progress by site directed mutagenesis.

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