Volume 280, number 1, 163–166 FEBS 09481 March 1991

ADONIS 001457939100191T

Tyr-426 of the *Escherichia coli* asparaginyl-tRNA synthetase, an amino acid in a C-terminal conserved motif, is involved in ATP binding

Jocelyne Anselme and Michael Härtlein

European Molecular Biology Laboratory, 38042 Grenoble Cèdex, France

Received 20 December 1990; revised version received 17 January 1991

Sequence comparisons of the *E. coli* asparaginyl-tRNA synthetase (NRSEC) with aminoacyl-tRNA synthetase sequences of class II enzymes show significant homologies with aspartyl- and lysyl-tRNA synthetases. Three conserved regions were found, one of which is located in the C-terminal part of the NRSEC sequence. Site-directed mutagenesis was performed in this conserved region. A single point mutation Tyr-426→Ser results in a 15-fold increase in the K_m for ATP, while all the other kinetic parameters remain unchanged. The replacement of this Tyr-426 by a Phe does not affect the kinetic behaviour of the enzyme. These data indicate that Tyr-426 is part of the ATP binding site.

Sequence homology; Aminoacyl-tRNA synthetase; Site-directed mutagenesis; ATP binding

1. INTRODUCTION

Aminoacyl-tRNA synthetases are a group of enzymes which catalyse the attachment of amino acids to their cognate tRNA molecules (see [1] for review). They vary greatly both in size and in quaternary structure, and show very limited sequence homology. They are however small conserved regions, some of which are known to have functional importance. The existence of these sequences allow the grouping of all synthetases into two classes [2,3].

The class I enzymes contain two sequence motifs, His-Ile-Gly-His (HIGH) which form part of the binding site for ATP [4,5], and Lys-Met-Ser-Lys-Ser (KMSKS) which has been shown by cross-linking to be close to the 3′-end of the tRNA [6,7]. The class II enzymes do not have these sequences, but have three other conserved motifs [2,8,9]. This class includes asparaginyl-, aspartyl-, histidyl-, lysyl-, prolyl-, seryl- and threonyl-tRNA synthetases, and the β subunit of phenylalanyl-tRNA synthetase. Recently the first three-dimensional structure of a class II enzyme was described, namely seryl-tRNA synthetase from *E. coli* (SRSEC) [3]. The catalytic domain is based on a seven-stranded antiparallel β-sheet which contains two of the three conserved motifs.

The *ansS* gene coding for the *E. coli* asparaginyl-tRNA synthetase had been previously cloned and sequenced [9,10]. Site-directed mutagenesis experiments were performed in the C-terminal conserved region. We describe here a single point mutant which exhibit a large increase in the K_m value for ATP, with the other kinetic parameters being unchanged. These data show the importance of this amino acid as part of the ATP binding site.

Correspondence address: J. Anselme, European Molecular Biology Laboratory, Grenoble Outstation, 156 X, 38042 Grenoble Cédex, France

Abbreviations: FPLC, fast protein liquid chromatography; k_{cat}, catalytic rate constant; K_m, Michaelis constant; SDS, sodium dodecyl sulphate

2. MATERIALS AND METHODS

2.1. *Sequence comparison among aminoacyl-tRNA synthetases*

The sequence of NRSEC was aligned with several aminoacyl-tRNA synthetases using the program BESTFIT of the University of Wisconsin, Genetics Computer group [11], using a gap weight of 2 and a gap length of 0.05.

2.2. *Construction of vectors expressing native and mutated NRSEC*

The *asnS* gene was previously cloned and sequenced [9,10]. A 1.1-kb *Eco*RI-*Bgl*II fragment carrying the 3′-end of the *asnS* gene was subcloned into M13mp19. Site-directed mutagenesis was performed following the method of Eckstein and co-workers [12], using the oligonucleotide: 5′ CGATCTGCGTCGCTA(C,T)CGGTACTG-TTC(G,T)CATTCAGGTTTCGGTCTTGGTTT 3′. The oligonucleotide was synthesised on an Applied Biosystem Apparatus 380.B at EMBL, Heidelberg and subsequently purified by reverse-phase chromatography. Except at the three positions which are underlined, this oligonucleotide is identical to the region 1263–1313 (position 1 is the A of the AUG initiation codon) of the coding strand for NRSEC. The bases 1277 and 1288 in the oligonucleotide are composed of a mixture of the wild type base (80%) and of two other bases (in brackets, 10% each). At position 1290, the base G is replaced by a T, resulting in formation of an *Eco*NI site.

Single-stranded DNA was sequenced according to the protocol of Sanger et al. [13], using a synthetic primer identical to positions 1175–1190 of the coding strand of the *asnS* gene.

A 1.3-kb *Mlu*I-*Sph*I mutated fragment isolated from the replicative form of the M13mp19 construction was used to replace the homologous fragment in an *asnS* gene cloned and expressed in the vector pUC18.

The replacement of the native fragment by the mutated homologue

was analysed by *Eco*NI digestion of the resulting plasmids, followed by sequencing of the pertinent region.

The native and mutated enzymes were purified from 17 litres of bacterial culture, in 3 fractionation steps following the scheme of Leberman et al. [14], except that the last purification step was performed by anion-exchange on a FPLC Mono Q HR 10/10 column (Pharmacia).

2.3. *Kinetic analysis of the purified enzymes*

ATP-PP_i exchange and aminoacylation assays were performed for both native and mutated purified enzymes, according to Calendar and Berg [15], and Jakes and Fersht [16]. The enzymes were at least 95% pure, as judged by electrophoresis on SDS-polyacrylamide gels.

The activation reaction was performed in a buffer consisting of 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 0.46 mM [^{32}P]PP_i (10 cpm/picomol), containing various amounts of the substrate studied (asparagine or ATP) while the other substrate was kept saturating. Typical concentrations of the substrate studied were from 0.1 to 10 times K_m. Saturating concentration of the other substrate was 53 μM asparagine or 2 mM Mg-ATP (molecular ratio 1:1), except in the case of the mutant Tyr-426Ser, where the ATP concentration used was 10 mM. The enzyme concentration was 120 nM.

The charging activity was measured in a buffer consisting of 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 2×10^{-4} M Spermidine, 11.2 mg/ml crude tRNA (Boehringer Mannheim). This corresponded to 4 μM $tRNA^{asn}$. Concentrations of the substrate studied ([^{14}C]asparagine, 100 cpm/picomol, or Mg-ATP) were from 0.1 to 2 times K_m. Concentration of the other substrate was 53 μM [^{14}C]asparagine (100 cpm/picomol) or 2 mM Mg-ATP. Enzyme concentration was 23 nM.

3. RESULTS

As described previously [2,9], NRSEC shows a remarkable sequence homology with aspartyl- and lysyl-tRNA synthetases (29% and 23%, respectively, using the BESTFIT program as described in section 2). An interesting homology is found with a surface protein of unknown role from *Brugia malayi* (23%) [17], which indictes that this protein may be an aminoacyl-tRNA synthetase.

NRSEC	420	Y	R	D	L	R	R	**Y**	G	T	V	P	H	S	G	F	G	L	G	F	E	R	L	441
DRSEC	517	L	L	D	A	L	K	**Y**	G	T	P	P	H	A	G	L	A	F	G	L	D	R	L	538
DRSSCM	587	L	L	N	A	F	D	**M**	G	T	P	P	H	A	G	F	A	I	G	F	D	R	M	608
DRSSC	511	Y	C	D	G	F	S	**Y**	G	C	P	P	H	A	G	G	G	I	G	L	E	R	V	532
DRSRL	428	Y	I	D	S	F	R	**F**	G	A	P	P	H	A	G	G	G	I	G	L	E	R	V	449
DRSHS	454	Y	I	D	S	F	R	**F**	G	A	P	P	H	A	G	G	G	I	G	L	E	R	V	475
KRSECu	461	Y	V	T	A	L	E	**Y**	G	L	P	P	T	A	G	L	G	I	G	I	D	R	M	482
KRSECs	461	Y	V	T	A	L	E	**H**	G	L	P	P	T	A	G	L	G	I	G	I	D	R	M	482
KRSSC	535	F	C	N	A	L	E	**Y**	G	L	P	P	T	G	G	W	G	C	G	I	D	R	L	556
ANTBM	502	Y	L	D	Q	R	L	**Y**	G	T	C	P	H	G	G	Y	G	L	G	L	E	R	F	523
								*			*			*				*			*			
Consensus Sequence		Y	.	D	.	.	.	Y	G	.	P	P	H	A	G	.	G	I	G	L	Y	R	Ø	

Fig. 1. A conserved region close to the C-terminal end of the NRSEC. Sequence comparisons were performed with the program BESTFIT according to Smith and Waterman [11], using standard conditions. Tyrosine-426 of the *E. coli* asparaginyl-tRNA synthetase which was mutagenised (this article) is in bold in this Figure. Amino acids which are totally conserved among all compared sequences are indicated by stars. Ø, hydrophobic residues, Y, aspartic or glutamic acids. NRSEC, *E. coli* asparaginyl-tRNA synthetase [9]; DRSEC, *E. coli* aspartyl-tRNA synthetase [18]; DRSSCM, yeast mitochondrial aspartyl-tRNA synthetase [19]; DRSSC, yeast aspartyl-tRNA synthetase [20]; DRSRL, rat liver aspartyl-tRNA synthetase [21]; DRSHS, Human aspartyl-tRNA synthetase [22]; KRSECu; *E. coli* thermoinducible lysyl-tRNA synthetase [23]; KRSECs, *E. coli* constitutive lysyl-tRNA synthetase [24]; KRSSC, yeast lysyl-tRNA synthetase [25]; ANTBM, *Brugia malayi* 60 kd-antigen [17].

Fig. 1 shows a particularly well conserved region, close to the C-terminus of the NRSEC, with tyrosine-426 shown in bold. Amino acids at this position are often tyrosine (6/10), or if not, an aromatic residue (8/10). Five totally conserved amino acids (one proline, one arginine and three glycines), indicated by stars in Fig. 1, are present at positions 427, 430, 433, 437, 440 of the NRSEC. Tyr-426 was chosen for site-directed mutagenesis, as its substitution is less drastic as compared to that of glycine or proline [26].

The degenerated oligonucleotide used to perform site-directed mutagenesis was constructed in order to increase the yield of single point mutants. Screening was performed by sequencing the M13 DNAs in the region which was mutagenised. The average yield of mutants was 85%, with half of the mutants being single point substitutions. Replacement of the native wild type fragment by the mutated homologous fragment was screened by *Eco*NI digestion, and subsequently confirmed by sequencing of the mutated plasmid. This procedure allowed the isolation of two single point mutants, Y426F and Y426S.

As judged by SDS-polyacrylamide gel, the expression of the mutated enzymes represented respectively for Y426F and for Y426S, 10% and 6% of the total cytosoluble protein. Under these conditions, contamination by the chromosome encoded NRSEC was negligible (less than 0.2%, data not shown).

Tables I and II show the kinetic parameters for NRSEC and the two mutants. The K_m value for asparagine was not greatly modified when amino acid 426 was changed to either phenylalanine or serine. In contrast, when the Tyr-426 was replaced by serine, the K_m value for ATP was increased by a factor of 15 for the activation reaction, and by a factor of 3 in the overall charging reaction. This can be explained if the binding of the tRNA stabilizes the ATP binding site in an active conformation, and this stabilization might balance the loss of affinity for ATP in the mutated enzyme. The replacement of the same amino acid by

Table I

Kinetic parameters of the wild-type *E. coli* asparaginyl-tRNA synthetase and two mutants in the ATP-PP_i exchange reaction

Enzyme	Substrate studied	K_m	k_{cat} (s^{-1})
WT	asparagine	15.3 μM	2.8
Y426F	asparagine	22.5 μM	2.7
Y426S	asparagine	38.3 μM	1.3
WT	ATP	0.5 mM	2.2
Y426F	ATP	0.5 mM	2.3
Y426S	ATP	7 mM	2.1

Kinetic experiments were performed as described in section 2. Y426F, Y426S: tyrosine-426 of the native NRSEC was replaced respectively by phenylalanine and serine.

Table II

Kinetic parameters of the wild-type *E. coli* asparaginyl-tRNA synthetase and two mutants in the aminoacylation reaction.

Enzyme	Substrate studied	K_m	k_{cat} (s^{-1})
WT	asparagine	32.3 μM	1.6
Y426F	asparagine	32.6 μM	2.8
Y426S	asparagine	33.6 μM	0.7
WT	ATP	76 μM	1.3
Y426F	ATP	30.6 μM	1.0
Y426S	ATP	245 μM	0.3

Aminoacylation assays were performed according to Jakes and Fersht [16], under the conditions described in section 2. Y426F and Y426S: two mutants of NRSEC, with tyrosine-426 replaced by phenylalanine (F) or serine (S).

phenylalanine had little effect. Changes in k_{cat} values for both mutants were not significant.

In order to see whether the enzymes have the same stability at the temperature used to determine kinetic constants, we measured the rate of the activation reaction for all enzymes after one, two and six hours incubation at 37°C. As no significant changes were observed, we concluded that the stability of the enzymes at the temperature used for the experiments was unaffected by the mutations. As only one kinetic parameter was significantly modified, we could expect that the mutation did not induce a global modification of the enzyme structure.

4. DISCUSSION

According to recent results, aminoacyl-tRNA synthetases can be grouped into two classes [2,3]. The first class is now well characterised, as the three-dimensional structures of *Bacillus stearothermophilus* tyrosyl-tRNA synthetase (YRSBS), *E. coli* methionyl-tRNA synthetase, and recently *E. coli* glutaminyl-tRNA synthetase are known [27–29]. These 3 enzymes display significant structural homology, having in common a Rossmann dinucleotide binding fold [5,28,29]. In particular a β-α-β region containing the HIGH motif is found to be highly homologous. The existence of the conserved region HIGH in isoleucyl-, methionyl-, tyrosyl- and glutaminyl-tRNA synthetases has been shown by Webster et al. [4]. The structure of the complex between YRSBS and tyrosyl-adenylate [5], and extensive site-directed mutagenesis [30], have allowed the identification of the amino acids on the enzyme responsible for the binding of ATP. These include the histidines of the HIGH region. Subsequently, mutations in this conserved region were performed in a homologous enzyme, namely valyl-tRNA synthetase from *Bacillus stearothermophilus*, [31], also confirming in this case, the role of this region as an ATP binding site.

An *E. coli* strain carrying a mutation in the *argS* gene coding for arginyl-tRNA synthetase (RRSEC) shows a point mutation two amino acids downstream from the HIGH sequence [32]. This mutated RRSEC exhibits a 5-fold increase in the K_m for ATP, whereas the other kinetic parameters are not significantly changed.

A conserved region found between *E. coli* and yeast isoleucyl-tRNA synthetases, 26 amino acids after the HIGH sequence, was mutagenised [33]. A specific and large increase in the K_m for isoleucine was found, showing the importance of this region in the amino acid binding. Thus the conserved regions have been shown to have functional importance.

Among class II synthetases, the structure of the *E. coli* seryl-tRNA synthetase has been recently solved [3]. The putative active site does not contain the nucleotide binding fold as in class I enzymes, but is based on a seven-stranded antiparallel β-sheet. In class I enzymes, the ATP binding site is close to the N-terminus (for review see [1]), whereas for those of class II the putative site is located in the C-terminal part [3,22]. Based on sequence homology, the class II enzymes can be subdivided into a group (IIa) consisting of seryl-, threonyl-, prolyl- and histidyl-tRNA synthetases, and another one (IIb) of asparaginyl-, aspartyl- and lysyl-tRNA synthetases (Cusack et al., submitted to Embo J.). According to this sub-classification, there are 3 conserved regions of which the third motif can be roughly divided into two parts: the N-terminal segment, rich in glycine, is found only in the class IIb enzymes (asparaginyl-, aspartyl- and lysyl-tRNA synthetases). The C-terminal segment of this motif is common to all enzymes of class II and contains the Gly-Leu-Glu-Arg highly conserved sequence [8,22].

Tyr-426 of NRSEC, in the N-terminal segment of the third motif, is situated just before helix H12 and β strand A5 of SRSEC, both thought to form part of the active site [3].

Site-directed mutagenesis has been performed on the *E. coli* aspartyl-tRNA synthetase (DRSEC) [18], the *E. coli* enzyme which presents the highest degree of homology with NRSEC. Leu-535 of DRSEC, which corresponds to Phe-438 of NRSEC, was replaced by proline. This mutant shows a decreased V_{max}, and an increased K_m (by a factor of 20 for the ATP, a factor of 2 for aspartic acid and $tRNA^{Asp}$). Due to the drastic nature of the substitution [26], one cannot exclude that the tertiary structure has been affected. The residue corresponding to Tyr-426 mutated here is positioned 12 amino acids ahead of Leu-535 of DRSEC.

This could support the idea that the third conserved region in class IIb enzymes is part of the ATP binding site.

A more complete set of mutants in this region, especially affecting the fully conserved amino acids, have to be obtained in order to better define residues which are directly involved in ATP binding. In the HIGH motif of class I enzymes, glycine does not play a

direct role in binding or catalysis, but promotes formation of the proper structure [6]. Some of the conserved glycines in the class II enzyme may play a similar role.

Acknowledgements: We thank Phillipe Neuner for the construction and purification of the mutagenic oligonucleotide, Dr Marc Delarue for communicating to us the sequence homology between NRSEC and the *Brugia malayi* protective antigen. We would like to thank Dr Reuben Leberman for helpful discussions and critical review of the manuscript.

REFERENCES

[1] Schimmel, P. (1987) Annu. Rev. Biochem. 56, 125–158.
[2] Eriani, G., Delarue, M., Poch, O., Gangloff, J. and Moras, D. (1990) Nature 347, 203–206.
[3] Cusack, S., Berthet-Colominas, C., Härtlein, M., Nassar, N. and Leberman, R. (1990) Nature 347, 249–255.
[4] Webster, T., Tsai, H., Kula, M.A., Mackie, G. and Schimmel, P. (1984) Science, 226, 1315–1317.
[5] Brick, P., Bhat, T.N. and Blow, D.M. (1988) J. Mol. Biol. 208, 83–98.
[6] Burbaum, J.J., Starzyk, R.M. and Schimmel, P. (1990) Proteins 7, 99–111.
[7] Hountondji, C., Dessen, P. and Blanquet, S. (1986) Biochimie 68, 1071–1078.
[8] Lévêque, F., Plateau, P., Dessen, P. and Blanquet, S. (1989) Nucleic Acids Res. 18, 305–312.
[9] Anselme, J. and Härtlein, M. (1989) Gene 84, 481–485.
[10] Tommassen, J., van der Ley, P., van der Ende, A., Bergmans, H. and Lugtenberg, B. (1982) Mol. Gen. Genet. 185, 105–110.
[11] Smith, T.F. and Waterman, M.S. (1981) Adv. Appl. Math. 2, 482–489.
[12] Nakayame, K. and Eckstein, F. (1986) Nucleic Acids Res. 24, 9679–9698.
[13] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[14] Leberman, R., Antonsson, B., Giovanelli, R., Guariguata, R., Schumann, R. and Wittinghofer, A. (1980) Anal. Biochem. 104, 29–36.
[15] Calendar, R. and Berg, P. (1966) Biochemistry 5, 1681–1690.
[16] Jakes, R. and Fersht, A.R. (1975) Biochemistry 14, 3344–3350.
[17] Nilsen, T.W., Maroney, P.A., Goodwin, R.G., Perrine, K.G., Denker, J.A., Nanduri, J. and Kazura, J.W. (1988) Proc. Natl. Acad. Sci. USA 85, 3604–3607.
[18] Eriani, G., Dirheimer, G. and Gangloff, J. (1990) Nucleic Acids Res. 18, 7109–7118.
[19] Gampel, A. and Tzagoloff, A. (1989) Proc. Natl. Acad. Sci. USA 86, 6023–6027.
[20] Sellami, M., Charton, B., Fasiolo, F., Dirheimer, G., Ebel, J.P. and Gangloff, J. (1986) Nucleic Acids Res. 14, 1657–1666.
[21] Mirande, M. and Waller, J.P. (1989) J. Biol. Chem. 264, 842–847.
[22] Jacobo-Molina, A., Peterson, R. and Yang, D.C.H. (1989) 264, 16608–16612.
[23] Clark, R.L. and Neidhardt, F.C. (1990) J. Bacteriol. 172, 3237–3243.
[24] Kawakami, K., Jönsson, Y.H., Björk, G.R., Ikeda, H. and Nakamura, Y. (1988) Proc. Natl. Acad. Sci. USA 85, 5620–5624.
[25] Mirande, M. and Waller, J.P. (1988) J. Biol. Chem. 263, 18443–18451.
[26] Pakula, A.A. (1989) Annu. Rev. Genet. 23, 289–310.
[27] Brick, P. and Blow, D.M. (1987) J. Mol. Biol. 194, 287–297.
[28] Zelwer, C., Risler, J.L. and Brunie, S. (1982) J. Mol. Biol. 155, 63–81.
[29] Rould, M.A., Perona, J.J., Söll, D. and Steitz, T.A (1989) Science 246, 1135–1141.
[30] Jones, M.D., Lowe, D.M., Borgford, T. and Fersht, A.R. (1986) Biochemistry 25, 1887–1891.
[31] Borgford, T.J., Gray, T.E., Brand, N.J. and Fersht, A.R. (1987) Biochemistry 26, 7246–7250.
[32] Eriani, G., Dirheimer, G. and Gangloff, J. (1990) Nucleic Acids Res. 18, 1475–1479.
[33] Clarke, N.D., Lien, D.C. and Schimmel, P. (1988) Science 240, 521–523.