Volume 293, number 1,2, 160–163 FEBS 10412 November 1991

Impaired affinity for phenylalanine in *Escherichia coli* phenylalanyl-tRNA synthetase mutant caused by Gly-to-Asp exchange in motif 2 of class II tRNA synthetases

Peter Kast, Claudia Wehrli and Hauke Hennecke

Mikrobiologisches Institut, Eidgenössische Technische Hochschule, Schmelzbergstrasse 7, CH-8092 Zürich, Switzerland

Received 23 September; revised version received 1 October 1991

Phenylalanyl-tRNA synthetase (PheRS; $\alpha_2\beta_2$ subunit structure) is a member of class II of tRNA synthetases. We report here the genetic analysis of an *Escherichia coli* mutant strain which is auxotrophic for phenylalanine because it has a PheRS with a decreased affinity for phenylalanine. The mutant *pheS* gene encoding the PheRS α subunit was cloned and sequenced, and the deviation from the wild-type gene was found to result in a Gly^{191}-to-Asp^{191} exchange. This alteration is located within motif 2, one of 3 conserved sequence motifs characteristic for class II aminoacyl-tRNA synthetases. Motif 2 may thus participate in the formation of the phenylalanine binding site in PheRS.

Amino acid binding site; Class II aminoacyl-tRNA synthetase; K_m mutant; Phenylalanine auxotrophy; Phenylalanyl-tRNA synthetase; *pheS* gene mutation

1. INTRODUCTION

Aminoacyl-tRNA synthetases catalyze the coupling of amino acids to their corresponding tRNAs, a key reaction in the transmission of the genetic information [1]. Recently, all aminoacyl-tRNA synthetases were subdivided into 2 non-related classes [2,3] based on sets of characteristic consensus sequence motifs [4,5 (class I); 2,6 (class II)]. The class I tRNA synthetase consensus sequences (HIGH [7] and KMSKS [8]) are known to carry the catalytic and the substrate binding sites, as deduced from analyses of mutants [4,9] and from the available tertiary structures of MetRS [10], TyrRS [11] and GlnRS [12]. Of the class II tRNA synthetases, 2 crystal structures were published recently (*Escherichia coli* SerRS [13] and yeast AspRS [14]). The comparison of these 2 structures revealed the presence of a common active site domain [14] which is unrelated to the corresponding catalytic domain of class I tRNA synthetases [4,12], and which contains the 3 conserved class II tRNA synthetase consensus motifs discovered before [6,14]. However, since co-crystals of SerRS or AspRS with their amino acid substrates were not available for X-ray analyses, a structure-function correlation between amino acid binding and conserved motifs of class II tRNA synthetases remains to be established.

The approach used in our laboratory to localize possible determinants of the Phe binding site in *E. coli* phenylalanyl-tRNA synthetase (PheRS, a class II enzyme) consists of the analysis of PheRS mutants with altered substrate binding properties. In a previous report, we deduced from the analysis of a PheRS mutation leading to *p*-fluoro-phenylalanine resistance that the class II tRNA synthetase consensus motif 3 is involved in Phe binding [15]. In the present work, a PheRS mutant with an increased K_m for Phe was examined. The mutation caused Phe auxotrophy and slight thermosensitivity of *E. coli* mutant strain G1 [16]. The mutation was previously [16] mapped to *pheS*, the gene encoding the (small) α subunit of PheRS. Therefore, we cloned and sequenced the mutant *pheS* gene of strain G1 to identify the resulting amino acid exchange.

Abbreviations: bp, basepair(s); IPTG, isopropyl-β-D-thiogalactopyranoside; kb, kilobasepair(s); K_m, Michaelis–Menten constant; ts, thermosensitive; XxxRS, aminoacyl-tRNA synthetase for amino acid Xxx in 3-letter-code.

Correspondence address: H. Hennecke, Mikrobiologisches Institut, Eidgenössische Technische Hochschule, Schmelzbergstrasse 7, CH-8092 Zürich Switzerland. Fax: (41) (1) 262 0647.

2. EXPERIMENTAL

2.1. *Bacterial strains, vectors and plasmids*

The *E. coli* PheRS mutant strains used were: the ethylmethanesulfonate-mutagenized Phe auxotrophic strain G1 (*pheS76* [16]) possessing a slightly thermosensitive PheRS with an increased K_m for Phe; the $PheRS^{ts}$ mutant strain NP37 (*pheS5* [17–19]); and strain KA2, a tetracyclin-resistant and RecA⁻ derivative of NP37 constructed by P1 transduction [15]. Strains JM109 and JM101 [20] served as hosts for cloning and single-strand DNA isolation, respectively. All strains were usually grown in LB-medium [21]. The vectors pUC19 [20] and pBluescript KS(+) (=pBLS [22]) were purchased from Pharmacia (Uppsala, Sweden) and Stratagene (San Diego, CA, USA), respectively. Plasmids pKSC-W, pKSB1-W and pKSB2-W have been described in detail [15]; they all contain *pheS* genes from a wild-type *E. coli* strain.

 Published by Elsevier Science Publishers B.V.

2.2. *Cloning of the mutant* pheS *gene*

The mutant *pheS* gene from *E. coli* strain G1 was cloned on a 10.25 kb *Eco*RI/*Hin*dIII fragment carrying the genes *thrS*, *infC*, *rpmI*, *rplT*, *pheS* and *pheT* [15,23] by applying the procedure described recently [15]. Briefly, chromosomal DNA from strain G1 was digested with *Eco*RI and *Hin*dIII and checked by Southern-blot analysis for the presence of the 10.25 kb *Eco*RI/*Hin*dIII fragment carrying *pheS*. Then, DNA fragments of the 10 kb size range were isolated from a preparative agarose gel and ligated to pUC19 vector digested with *Eco*RI and *Hin*dIII. After transformation of the thermosensitive *E. coli* strain NP37 (*pheS*^{ts}) with these pUC19-based plasmids, incubation at high temperature (40°C) selected for NP37 cells that contained a plasmid (pKSC-G) carrying the PheRS genes of strain G1.

2.3. *Subcloning and sequencing strategy*

The mutant *pheS* gene from plasmid pKSC-G was subcloned by inserting the *pheS*-containing 1181 bp *Sma*I/*Hin*dII fragment of pKSC-G in both orientations into the *Sma*I site of the single-strand sequencing vector pBluescript KS(+) [15]. The resulting constructs pKSB1-G and pKSB2-G allowed the overlapping sequencing of the mutant *pheS* gene from strain G1 in parallel with that of the wild type (on the plasmids pKSB1-W and pKSB2-W) on both strands by using *pheS*-specific oligonucleotide primers. The sequencing strategy and methods employed were detailed elsewhere [15,24].

2.4. *Plate tests: in vivo phenotypic analysis*

In vivo plate tests for complementation of strain KA2 were performed by streaking out single colonies on LB-agar plates containing ampicillin at 150 μg/ml. IPTG (Bachem AG, Switzerland) was present at 0.5 mM to fully express *pheS* on plasmids pKSB1-G and pKSB1-W (*pheS* in phase with the *lac* promoter). Growth was recorded after 24 and 48 h of incubation at 30°C, 36°C or 40°C. All tests were performed in duplicate and repeated at least once.

3. RESULTS AND DISCUSSION

3.1. *Cloning and sequencing of* pheS *from strain G1*

To determine the mutation responsible for the increased K_m of PheRS in the Phe auxotrophic *E. coli* strain G1, the mutant *pheS* gene was cloned, subcloned and sequenced as detailed in sections 2.2. and 2.3. The only deviation between *pheS* of strain G1 and the wild-type sequence [15,23] consisted of a G-to-A transition at nucleotide position 572 (Fig. 1) leading to a Gly-to-Asp exchange at position 191 in the α subunit (mutation D191). This alteration is obviously responsible for the reported phenotypes of strain G1 [16].

3.2. *In vivo phenotype of the cloned* pheS *mutant gene in a thermosensitive (*pheS^{ts}*) host strain*

To verify that the cloned *pheS* gene mutation corresponds to that of strain G1, we analyzed the phenotype caused by the *pheS* expression plasmid pKSB1-G. Consistent with the slight thermosensitivity of strain G1 [16], expression of *pheS* from plasmid pKSB1-G complemented the thermosensitive strain KA2 (*pheS*^{ts}) only marginally on non-supplemented LB medium (Table I). It was observed previously with several thermosensitive aminoacyl-tRNA synthetase mutants having impaired substrate interactions that the thermosensitive phenotype could be (partially) rescued by increasing the corresponding substrate concentrations [25,26]. Similarly, Phe supplementation completely reversed the thermosensitive trait of strain G1 (data not shown) and partially that of strain KA2 harbouring pKSB1-G (Table I). This was further proof that the cloned *pheS* gene originated from the Phe auxotrophic strain G1.

Although *pheS* from strain G1 could not complement strain KA2 (*pheS*^{ts}) at 40°C (Table I), the G1 allele was initially cloned by complementation of the same *pheS*^{ts} mutation. The apparent paradox is explained by an increased copy number of the rather inefficient PheRS of strain G1, due to the simultaneous presence of both PheRS subunit genes *pheS* and *pheT* on the initial plasmid pKSC-G. In vivo tests with pKSC-G and pKSC-W (carrying wild-type *pheST*) showed that both plasmids were indistinguishable in their ability to complement the thermosensitivity of strain KA2 (data not shown). Other examples in which an increase in the copy number of a defective aminoacyl-tRNA synthetase reversed a thermosensitive phenotype have been described [16,27,28].

3.3. *Mutation D191 lies within a conserved sequence motif of class II tRNA synthetases*

As a member of class II aminoacyl-tRNA synthetases, the PheRS α subunit contains at least 2 of the 3 conserved sequence motifs which define this group [2]. The presence of motif 1 assumed to be involved in the subunit interface in the dimeric (α_2) class II tRNA synthetases [13,14] has been questioned recently for the

Table I

In vivo phenotype of the cloned *pheS* gene from strain G1

Strain/plasmid	Presence of PheRS allele on plasmid[b]	Growth on agar plates[a]					
		LB, ampicillin, IPTG			LB, ampicillin, IPTG+L-Phe (3 mg/ml)		
		30°C	36°C	40°C	30°C	36°C	40°C
KA2/pBLS	-	++	0	0	++	0	0
KA2/pKSB1-W	*pheS*	++	++	++	++	++	++
KA2/pKSB1-G	*pheS76*	++	+	0	++	+	+

[a]Growth of single colonies is specified from good growth to no (or only marginal) growth, in the order: ++>++>+>0

[b]*pheS* and *pheS76* encode the α subunits of PheRS from the wild type and strain G1, respectively

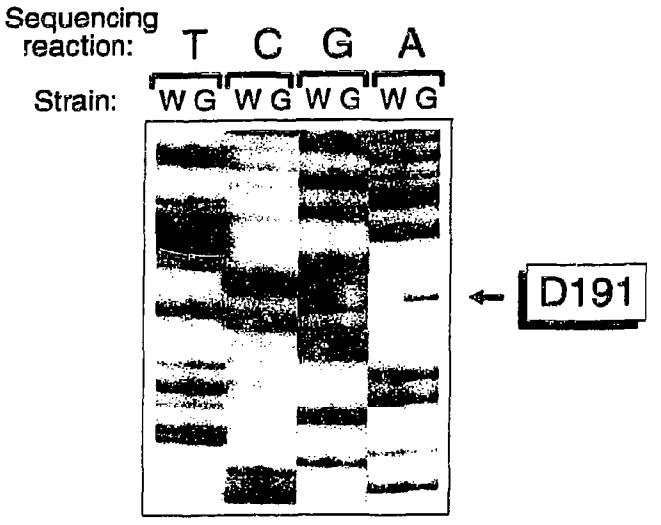

Fig. 1. The point mutation identified in *pheS* from strain G1. The autoradiogram shows the sequences around nucleotide position 572. The sequencing reactions for the same base specificity were loaded in adjacent lanes in order to directly compare wild-type (W) and G1 (G) *pheS* sequencing patterns. Mutation D191 in strain G1, consisting of a G-to-A exchange, is indicated.

tetrameric PheRS [6]. Interestingly, the K_m mutation D191 (Gly-to-Asp) maps within motif 2, only 4 residues away from a universally conserved Arg [2]. Fig. 2 displays an alignment of the relevant known PheRS sequences and the motif 2 consensus. As shown in [6], a Gly is present at the position corresponding to the location of the mutation D191 in 9 out of 20 aligned class II tRNA synthetase wild-type sequences, whereas the yeast cytoplasmic PheRS (Fig. 2) happens to have an Asp residue there. In this context it is worth mentioning that the yeast enzyme displays a 5-fold higher K_m for Phe as compared to the *E. coli* enzyme [29]. The increase of K_m in D191 mutant PheRS (from strain G1) was 15-fold relative to the wild type [16]. Since an impaired Phe binding might be indicative for a mutation in the Phe binding site, it is tempting to propose, therefore, that mutation D191 identifies motif 2 as participating in the amino acid binding site in PheRS, and more generally in class II synthetases. This hypothesis is supported by several functional and structural considerations:

(i) Some functional evidence can be derived from mutagenesis experiments done with *E. coli* and yeast AspRS, which demonstrated the crucial importance of motif 2 residues for class II tRNA synthetase activity [2,30]. Of special interest is the proposed active site residue His^{334} in motif 2 of the yeast AspRS, which is 13 amino acids distant from the position corresponding to the PheRS mutation analyzed in this work; its replacement by the neutral amino acid Gln caused (among other effects) a 6-fold increase in the K_m for Asp, whereas an exchange against the more positively charged Arg resulted in a decrease of the K_m by a factor of 14 [30]. One explanation may be that residue 334 of yeast AspRS interacts with the negatively charged carboxyl group of the amino acid substrate.

That K_m mutations may actually reflect alterations in the corresponding substrate binding sites was already found for other aminoacyl-tRNA synthetase mutants [1,31–33]. An alteration in the probable amino acid binding site [10–12] was the cause for an elevated K_m for the cognate amino acid substrate and for thermosensitivity in a yeast MetRS mutant [27]. As shown here with a mutation responsible for a similar phenotype in PheRS, the identified amino acid replacement consisted of a Gly-to-Asp exchange. Thermosensitivity could result from decreased protein stability or from impaired interactions between the enzyme and its substrate; e.g. due to increased electrostatic repulsion as a consequence of the additional negative charge introduced by the amino acid exchange.

(ii) The possible involvement of motif 2 in amino acid substrate binding is further supported from a structural point of view. The active site domain found in both the

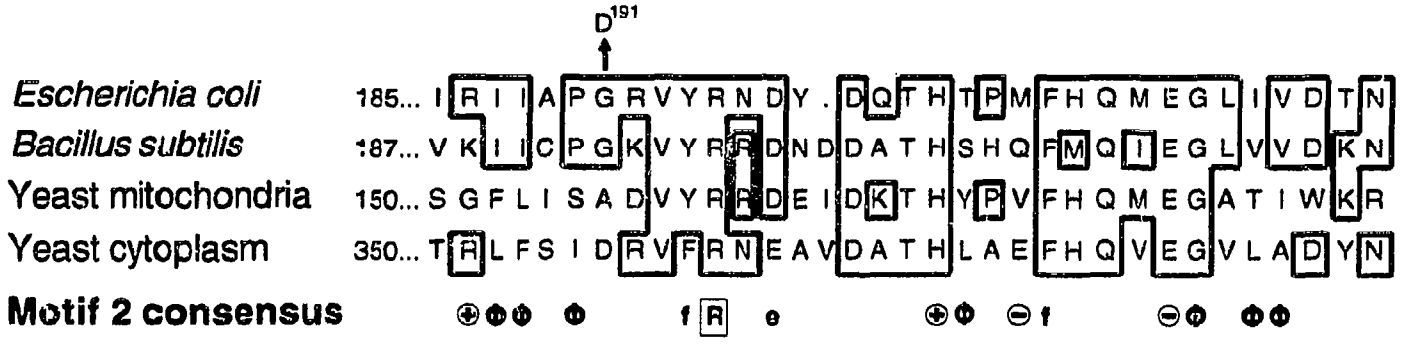

Fig. 2. Alignment of PheRS small subunit sequences encoded by the *pheS* region around the position of mutation D191. The region corresponding to the motif 2 consensus sequence [2] of the *pheS* gene products from *E. coli* [23], *B. subtilis* [35], yeast mitochondria [36] and yeast cytoplasm [37] are aligned. Numbers indicate the first residues shown here for the respective sequences. The one-letter code for amino acids is used. Identical residues are boxed. In addition, the motif 2 consensus sequence of class II synthetases as given in [2] is aligned. Lower case letters denote predominantly occurring residues; the boxed R, arginine, is completely conserved; Φ, hydrophobic amino acids; + and −, correspondingly charged residues. Detailed sequence alignments in the motif 2 region of more class II aminoacyl-tRNA synthetases are shown elsewhere [2,6].

SerRS [13] and AspRS [14] crystal structures, and which contains the conserved class II tRNA synthetase-specific sequence motifs, consists of a large central antiparallel β-sheet rimmed by loops [6,13,14]. It seems reasonable to assume that this super-secondary structure is the scaffold of the active site domain in all class II tRNA synthetases (including PheRS), as indicated by the presence of the conserved sequence motifs [6,14]. A clear assignment of general functions in catalysis and substrate binding to the individual class II motifs was only possible for a central loop within motif 2 which seems to recognize and bind the acceptor stem and the 3'-end of the bound tRNA, as deduced from the crystallized AspRS-tRNA^{Asp} complex [14]. Since the tRNA 3'-end must react with the carboxyl group of the amino acid substrate, a simultaneous involvement of motif 2 residues in binding the amino acid appears possible. For the residues corresponding to the PheRS α subunit around position 191, such a role would be compatible with their location in the first antiparallel strand of the large β-sheet [6,13,14].

In a previous report, we had proposed that residue 294 of the PheRS α subunit (within motif 3) might be a specificity determinant in the Phe binding site of PheRS [15]. Some of the mutants constructed in that work showed not only changes in the substrate specificity but also variations in the K_m values for Phe. We now suggest that residues from both motif 2 and motif 3 together might contribute to the amino acid substrate binding site in class II aminoacyl-tRNA synthetases. Again, this proposal is in agreement with the topology of the homologous sequences in the tertiary structures of SerRS [13] and AspRS [14]. The positions corresponding to residues 191 (motif 2) and 294 (motif 3) of the PheRS α subunit participate in individual β-strands at the bottom of the active site. Their relative topology and that of the afore-mentioned position 334 of AspRS (motif 2) seem to make possible a potential cooperation in amino acid binding [6,13,14]. Based on extensive sequence comparisons within class II tRNA synthetases, Cusack et al. [6] have proposed that the β-strand of motif 3 and 2 other, immediately adjacent β-strands, are involved in the specific binding of the amino acid substrate's side chain [6,34]. However, as already discussed previously [15], the situation in PheRS may be more complex because of the possible participation of the additional β subunit in the active site of PheRS.

An analysis of the recently obtained crystals of SerRS in the presence of ATP and serine [34] will perhaps help to elucidate the true topology of an amino acid binding site in a class II tRNA synthetase.

Acknowledgement: This work was supported by a grant from the Federal Institute of Technology Zürich.

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] Schimmel, P. (1991) Trends Biochem. Sci. 16, 1–3.
[4] Burbaum, J.J., Starzyk, R.M. and Schimmel, P. (1990) Proteins: Struct., Funct., Genet. 7, 99–111.
[5] Eriani, G., Dirheimer, G. and Gangloff, J. (1991) Nucleic Acids Res. 19, 265–269.
[6] Cusack, S., Härtlein, M. and Leberman, R. (1991) Nucleic Acids Res. 19, 3489–3498.
[7] Webster, T.A., Lathrop, R.H. and Smith, T.F. (1987) Biochemistry 26, 6950–6957.
[8] Hountondji, C., Dessen, P. and Blanquet, S. (1986) Biochimie 68, 1071–1078.
[9] Leatherbarrow, R.J., Fersht, A.R. and Winter, G. (1985) Proc. Natl. Acad. Sci. USA 82, 7840–7844.
[10] Brunie, S., Zelwer, C. and Risler, J.-L. (1990) J. Mol. Biol. 216, 411–424.
[11] Brick, P., Bhat, T.N. and Blow, D.M. (1989) J. Mol. Biol. 208, 83–98.
[12] Rould, M.A., Perona, J.J., Söll, D. and Steitz, T.A. (1989) Science 246, 1135–1142.
[13] Cusack, S., Berthet-Colominas, C., Härtlein, M., Nassar, N. and Leberman, R. (1990) Nature 347, 249–255.
[14] Ruff, M., Krishnaswamy, S., Boeglin, M., Poterszman, A., Mitschler, A., Podjarny, A., Rees, B., Thierry, J.C. and Moras, D. (1991) Science 252, 1682–1689.
[15] Kast, P. and Hennecke, H. (1991) J. Mol. Biol. 222, (in press).
[16] Grüll, J.M., Hennecke, H., Fröhler, J., Thomale, J., Nass, G. and Böck, A. (1979) J. Bacteriol. 137, 480–489.
[17] Eidlic, L. and Neidhardt, F.C. (1965) J. Bacteriol. 89, 706–711.
[18] Böck, A. and Neidhardt, F.C. (1967) Science 157, 78–79.
[19] Comer, M.M. and Böck, A. (1976) J. Bacteriol. 127, 923–933.
[20] Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103–119.
[21] Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[22] Short, J.M., Fernandez, J.M., Sorge, J.A. and Huse, W.D. (1988) Nucleic Acids Res. 16, 7583–7600.
[23] Fayat, G., Mayaux, J.-F., Sacerdot, C., Fromant, M., Springer, M., Grunberg-Manago, M. and Blanquet, S. (1983) J. Mol. Biol. 171, 239–261.
[24] Kast, P. (1991) Ph. D. thesis No 9468, Eidgenössische Technische Hochschule Zürich, Switzerland.
[25] Goodman, R. and Schwartz, I. (1988) Nucleic Acids Res. 16, 7477–7486.
[26] Chow, C.M., Metzenberg, R.L. and Rajbhandary, U.L. (1989) Mol. Cell. Biol. 9, 4631–4644.
[27] Chatton, B., Winsor, B., Boulanger, Y. and Fasiolo, F. (1987) J. Biol. Chem. 262, 15094–15097.
[28] Jasin, M. and Schimmel, P. (1984) J. Bacteriol. 159, 783–786.
[29] Gabius, H.-J., von der Haar, F. and Cramer, F. (1983) Biochemistry 22, 2331–2339.
[30] Gasparini, S., Vincendon, P., Eriani, G., Gangloff, J., Boulanger, Y., Reinbolt, J. and Kern, D. (1991) Biochemistry 30, 4284–4289.
[31] Fersht, A.R. (1987) Biochemistry 26, 8031–8037.
[32] Clarke, N.D., Lien, D.C. and Schimmel, P. (1988) Science 240, 521–523.
[33] Eriani, G., Dirheimer, G. and Gangloff, J. (1990) Nucleic Acids Res. 18, 1475–1479.
[34] Leberman, R., Härtlein, M. and Cusack, S. (1991) Biochim. Biophys. Acta 1089, 287–298.
[35] Brakhage, A.A., Wozny, M. and Putzer, H. (1990) Biochimie 72, 725–734 (Erratum (1991): Biochimie 73, 127).
[36] Koerner, T.J., Myers, A.M., Lee, S. and Tzagoloff, A. (1987) J. Biol. Chem. 262, 3690–3696.
[37] Sanni, A., Mirande, M., Ebel, J.-P., Boulanger, Y., Waller, J.-P. and Fasiolo, F. (1988) J. Biol. Chem. 263, 15407–15415.