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Asparaginyl-tRNA synthetase from the *Escherichia coli* temperature-sensitive strain HO202

A proline replacement in motif 2 is responsible for a large increase in K_m for asparagine and ATP

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The *Escherichia coli* K12 mutant gene, *asnS40*, coding for asparaginyl-tRNA synthetase (AsnRS) in the temperature-sensitive strain HO202, was isolated from genomic DNA using the Polymerase Chain Reaction. DNA sequencing revealed that the mutant enzyme differs from the wild-type AsnRS by two amino acids, but only the P231L replacement leads to a change in aminoacylation activity. In the ATP–PPi exchange reaction at 37°C the purified P231L enzyme has a more than 50-fold increased K_m value for asparagine compared to the wild-type enzyme, while the K_m value for ATP is increased 8-fold. In the aminoacylation reaction the mutant enzyme shows also significantly increased K_m values for asparagine and ATP. Interestingly Pro-231 is part of the conserved motif 2 in class II aminoacyl-tRNA synthetases (Eriani, G., Delarue, M., Poch, O., Gangloff, J. and Moras, D. (1990) Nature 347, 203–206), indicating that this motif might be involved in all class II enzymes in amino acid activation.

Asparaginyl-tRNA synthetase; Temperature-sensitive mutant; Amino acid binding; Class II aminoacyl-tRNA synthetase

1. INTRODUCTION

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

The class I enzymes contain two sequence motifs, His-Ile-Gly-His (HIGH) which forms part of the binding site for ATP [3,4], and Lys-Met-Ser-Lys-Ser (KMSKS) which has been shown by cross-linking to be close to the 3'-end of the tRNA [5,6]. Recently it has been shown that Lys-335, part of the KMSKS sequence, plays a crucial role in amino acid activation catalysed by the methionyl-tRNA synthetase from *E. coli* [7]. A substitution of this residue by glutamine renders the enzyme unable to stabilize the transition state complex in the methionine activation reaction.

The class II enzymes do not have these sequences, but have three other conserved motifs [1,8–10]. This class includes the alanyl-, asparaginyl-, aspartyl-, glycyl-, histidyl-, lysyl-, phenylalanyl-, prolyl, seryl- and threonyl-tRNA synthetases. The first three-dimensional structure of a class II enzyme solved was that of the seryl-tRNA synthetase from *E. coli* [11]. The overall topology of the catalytic domain is very close to that recently described for the yeast aspartyl-tRNA-synthetase, another class II enzyme [12]. This common domain is based on an anti-parallel-stranded β-sheet and contains the three conserved motifs: motif 1 is involved in the dimer interphase, and motifs 2 and 3 contribute to the formation of the catalytic cavity involved in the recognition of the amino acid, the ATP and the 3' CCA end of the tRNA. The crystal structure of the yeast aspartyl-tRNA synthetase with its cognate tRNA demonstrates the importance of a loop in sequence motif 2 for the interaction with the discriminator base and the first base pair of the acceptor stem.

The temperature-sensitive mutant, HO202, was isolated by H. Ohsawa and B. Maruo in 1976 and initially described by them as a mutant with an altered ribosomal protein S1 [13]. Later it was shown that the asparaginyl-tRNA synthetase and not S1 is responsible for the temperature-sensitivity [14]. HO202 has been used by Tommassen et al. (1982) to clone a DNA fragment carrying *asnS* and *ompF* genes by complementation with an *E. coli* gene bank DNA [15]. The DNA sequence of the *asnS* wild-type gene has been described [8].

We describe here the characterization of the gene for temperature-sensitive AsnRS in HO202 by PCR am-

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plification, cloning and sequencing, as well as the consequences of the determined amino acid changes on kinetic behaviour of the mutant enzyme.

2. MATERIALS AND METHODS

Unfractionated *E. coli* tRNA, bacterial alkaline phosphatase and T4 DNA ligase were purchased from Boehringer-Mannheim. 2′ deoxynucleoside 5′-triphosphates, DEAE Sepharose CL-4B and FPLC Mono Q HR 10/10 columns were from Pharmacia. L-[U-^{14}C]asparagine (228 mCi/mmol), [α-^{35}S]dATP (410 Ci/mmol), tetrasodium[^{32}P]pyrophosphate (47 mCi/mmol) and *Tub* DNA polymerase were from Amersham. Modified T7 DNA polymerase was from United States Biochemical. Geneclean II was from Bio101, USA. The oligonucleotide primers were synthesized on an Applied Biosystem Apparatus 381 A-00.

3. RESULTS

3.1. *Isolation of the mutant* asnS *gene by enzymatic amplification*

Genomic DNA of the *E. coli* K12 temperature-sensitive mutant, HO202, was prepared according to [16]. Synthesis of a 2 kb fragment containing the mutant asnS gene was conducted directly from its 3′ and 5′ ends by the PCR procedure in the presence of the appropriate 24-mer oligonucleotide primers. The reaction mixture (100 μl) contained 2 μg of genomic DNA, 50 mM TRIS–HCl, pH 9.0, 7.5 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 1 mM of each deoxynucleoside triphosphate, 2 U of *Tub* DNA polymerase and 1 μM of each primer. The mixture was overlaid with 100 μl of paraffin oil (Fluka) to prevent evaporation and transferred to a programmable heating block (PREM, LEP Scientific). After heating for 4 min at 94°C to denature the genomic DNA, the first primer annealing step was achieved at 35°C for 2 min. The bound primers were extended at 72°C for 2 min. This denaturation-annealing-extension cycle was repeated 30 times under the same conditions except that denaturation was performed for 2 min and annealing at 48°C. Electrophoretic analysis on a 1% agarose gel revealed a band of 2 kb corresponding to the expected distance between the primer annealing sites.

3.2. *Cloning and sequencing of the amplified fragment*

The 2 kb fragment was excised from a 1% agarose gel and purified with Geneclean II. A 1.55 kb fragment, containing the complete *asnS* coding sequence, was generated by *Ssp*I digestion, purified in the same way and cloned into pUC 18. Recombinant plasmids were then sequenced using appropriate oligonucleotides derived from the wild-type *asnS* DNA sequence.

Sequencing revealed that the HO202 *asnS* gene differs from the wild-type AsnRS structural gene by three mutations, all of which are substitutions of a cytosine by a thymine. One of these results in the substitution of Pro-231 by leucine, and an another one of Ala-266 by valine. The third mutation is in the wobble position of the arginine-375 codon. The two mutations leading to amino acid changes in AsnRS were cloned separately by recombining wild-type and mutant fragments, taking advantage of the occurrence of a *Eco*RI site between the two mutations and of a *Eco*O109I site in pUC 18.

3.3. *Aminoacylation activity at 30 and 45°C of transformant bacterial crude extracts*

Plasmids containing the wild-type *asnS* gene (pAsnS), the doubly mutated (pAsnS P231L, A266V) and the two singly mutated genes (pAsnS P231L and pAsnS A266V) were used to transform HO202.

Bacterial extracts were prepared and aminoacylation assays performed as described in [17]. Extracts from cells containing pAsnS p231L, A266V and pAsnS P231L have reduced activities at 30°C compared with the wild-type (42 and 21%, respectively), and strongly reduced activities at 45°C (4 and 3%, respectively). In contrast extracts from cells containing pAsnS A266V showed roughly wild-type activity (107% at 30°C and 111% at 45°C). From these results we concluded that the P231L mutation is mainly responsible for the phenotype of HO202 AsnRS. We studied therefore the kinetic behaviour of this mutant protein in more detail.

3.4. *Kinetic analysis of the purified enzymes*

The native and mutated P231L enzymes were purified from 2 l of bacterial culture, in three fractionation steps following the scheme of Lebermann et al. [18], except that the last purification step was performed by anion exchange on a FPLC Mono Q HR 10/10 column. They were at least 95% pure, as judged by electrophoresis on SDS-polyacrylamide gels. ATP–PPi exchange and aminoacylation assays were performed according to Calendar and Berg [19], and Jakes and Fersht [20].

The activation reaction was performed in a buffer consisting of 50 mM TRIS–HCl, pH 7.6, 10 mM $MgCl_2$, 5.5 mM [^{32}P]PPi (6.6 cpm/pmol), 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 0.1–10 × K_m. Saturating concentration of the other substrate was 3 mM asparagine or 8 mM MgATP. The enzyme concentration was 40 μM. The K_m and k_{cat} values are given in Table I. The mutant protein exhibits an increase in K_m for asparagine of more than 50-fold at 37°C, and 6-fold at 25°C (compared with the wild-type value at 37°C). The mutation significantly affected the K_m value for ATP at 37°C (8-fold increase). The k_{cat} values are quite similar for the mutant and wild-type proteins.

The charging activity was measured in a buffer consisting of 50 mM TRIS–HCl, pH 7.6, 10 mM $MgCl_2$, 0.2 mM spermidine and 12.5 mg/ml unfractionated tRNA corresponding to 4 μM $tRNA^{Asn}$. Concentrations of the substrate studied were from 0.1–5 × K_m. Concentration of the other substrate was 1.4 mM [^{14}C]asparagine (65

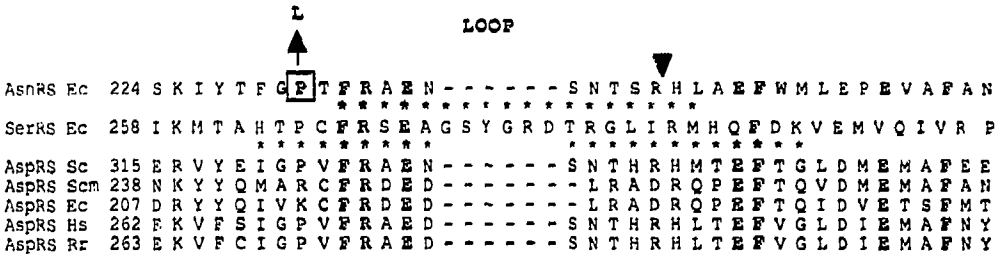

Fig. 1. Alignment of motif 2 of some class II aminoacyl-tRNA synthetases. Sequence comparisons were performed with the program BESTFIT according to Smith and Waterman [25], using standard conditions. Proline 231 of the *E. coli* asparaginyl-tRNA synthetase is boxed. The trypsine cleavage site is indicated by a triangle. Highly conserved residues are in bold. Loop regions in the structures of yeast aspartyl- [12] and *E. coli* seryl-tRNA synthetase [11] are marked with *. AsnRS Ec, *E. coli* asparaginyl-tRNA synthetase [8]; SerRS Ec, *E. coli* seryl-tRNA synthetase [26]; AspRS Scm, Sc, mitochondrial [27] and cytoplasmic [28] yeast aspartyl-tRNA synthetases; AspRS Ec, *E. coli* aspartyl-tRNA synthetase [22]; AspRS Hs, human aspartyl-tRNA synthetase [9]; AspRS Rr, rat liver aspartyl-tRNA synthetase [29].

cpm/pmol) or 8 mM MgATP. Enzyme concentration was 40 μM. The K_m for ATP increased 13-fold at 37°C. The increase in the K_m values for asparagine in the mutant protein relative to the wild- type protein (10-fold increase at 37°C, 3-fold at 25°C, see Table II) is smaller in the aminoacylation reaction than in the ATP–PPi exchange reaction. Differences in the k_{cat} values obtained by varying ATP or asparagine are stronger in the aminoacylation reaction than in the ATP–PPi exchange reaction. To obtain saturating conditions for asparagine we had to add unlabeled amino acid to the [^{14}C]asparagine. The low specific activity of the [^{14}C]asparagine mix might, to a certain extent, explain the differences in the k_{cat} values.

Differences in the K_m values measured in the exchange and aminoacylation reactions for the asparaginyl-tRNA synthetase have already been described and discussed in [21].

The P231L replacement in the mutant protein affects enzyme activity and stability at non-permissive temperatures for HO202 (42°C), but the enzyme is not irreversibly disorganized. Even after a pre-incubation at 50°C for 10 min the enzyme recovers full activity (measured at 25°C), whereas there is no detectable aminoacylation activity at 42°C.

4. DISCUSSION

The role of motif 3 in catalysis for class II aminoacyl-tRNA synthetases seems to be rather well defined. Site-directed mutagenesis of Tyr-426 in the *E. coli* asparaginyl-tRNA synthetase located at the beginning of motif 3 has shown that this residue has an important role in determining the kinetic parameters for ATP binding [21]. Site-directed mutagenesis has also been performed on the *E. coli* aspartyl-tRNA synthetase (AspRS) [22], the *E. coli* enzyme which presents the highest degree of homology with AsnRS. A replacement of Leu-535 in this motif by a proline leads to decreased V_{max} and a 20-times increased K_m value for ATP. Very recently Kast and Hennecke report the importance of Ala-294 (α subunit) for the amino acid substrate specificity of *E. coli* phenylalanyl-tRNA synthetase [23].

Less information is available concerning the role of the conserved motif 2. Very recently the functional importance of a histidine residue in motif 2 of yeast cytoplasmic aspartyl-tRNA synthetase has been demonstrated [24]. A replacement of this residue (H271) by a glutamine leads to a 10-fold decrease in the rate of the ATP–PPi exchange reaction together with a significant increase in the K_m values for the two small substrates, aspartic acid and ATP.

Table I

Kinetic parameters of the wild-type *E. coli* asparaginyl-tRNA synthetase and mutant P231L in the ATP–PPi exchange reaction

Enzyme	Substrate studied	K_m (μM)	k_{cat} (s^{-1})	Temperature (°C)
P231L	asparagine	94	3.0	25
WT	asparagine	15	2.8	37
P231L	asparagine	770	1.0	37
P231L	ATP	870	1.3	25
WT	ATP	500	2.2	37
P231L	ATP	4,000	2.0	37

Experiments were performed as described in Results.

Table II

Kinetic parameters of the wild-type *E. coli* asparaginyl-tRNA synthetase and mutant P231L in the aminoacylation reaction

Enzyme	Substrate studied	K_m (μM)	k_{cat} (s^{-1})	Temperature (°C)
WT	asparagine	29	2.8	25
P231L	asparagine	90	2.2	25
WT	asparagine	32	1.6	37
P231L	asparagine	330	0.9	37
WT	ATP	76	1.3	37
P231L	ATP	1,000	0.3	37

Experiments were performed as described in Results.

We described above a mutation in motif 2 of a class II enzyme using a different experimental approach. Its advantage compared to site-directed mutagenesis is that the changed genotype leads always to a modified enzyme activity (phenotype). A temperature-sensitive AsnRS mutant (HO202) of *E. coli* K12 has been reported, which has the interesting property of being able to grow in minimal medium at 40°C only in the presence of high concentrations of asparagine [14]. We identified the amino acid replacement responsible for the temperature-sensitive phenotype of HO202, which is a replacement of Pro-231 by a leucine. This residue is conserved in some of the class II synthetases including the *E. coli* serine enzyme, and is very near to a universally conserved motif 2 arginine (position 234 in AsnRS, see Fig. 1). We compared the kinetic parameters of the mutant enzyme with those of the wild-type enzyme in the ATP–PPi exchange and aminoacylation reactions. The most striking difference between the enzymes is in their affinity for the amino acid substrate; the mutant enzyme shows a large temperature-dependent increase in the K_m value for asparagine. However the fact that the affinity for the substrate ATP is also affected in a temperature-dependent fashion suggests that the P231 does not interact directly with the ligands, but has rather a structurating function.

The crystallographic structures of SerRS and AspRS show that part of motif 2 is present as a loop on the surface of the proteins. Limited trypsin digestion of AsnRS indicates that this is probably also the case for this enzyme: a stable peptide of M_r 26,000 is generated, the N-terminal sequence of which shows that it is produced by cleavage between residues R242 and H243, near P231 [8]. Accessibility of this region to trypsin indicates its location on the surface, and therefore it seems likely that a replacement of this residue induces only a local change in conformation of the motif 2 loop structure and not a global distortion of the enzyme.

This loop structure in the yeast AspRS interacts with the acceptor stem of the cognate tRNA in the major groove [12]. During catalysis, the 3′ end of the tRNA and the activated carboxyl function of the amino acid have to be close to each other. In the SerRS structure, the loop formed by part of the motif 2 might form a flexible lid over the active site region, which is stabilized on binding of substrates [11]. Due to its proximity, P265 of SerRS, homologous to P231 of AsnRS, could induce temperature-depending changes in conformations and dynamics of the loop, and thus modify the kinetic parameters of the enzyme. In the asparagine enzyme, the P231 replacement leads to a strongly decreased affinity for the substrates asparagine and ATP.

The fact that the mutant enzyme is not active at 42°C, but recovers its activity after heating to 42°C (or even to 50°C) and returning to 25°C, shows that the enzyme is not irreversibly inactivated. Matthews and co- workers [30] reported that substitutions of proline may cause a destabilization of proteins by increasing the entropy of unfolding. The backbone of proline has fewer accessible conformations in the unfolded state than other residues.

Our report describes a mutation of a residue in the conserved motif 2 which is responsible for an increase in K_m for both the amino acid and the ATP. Pro-231 does not seem to be implicated directly in substrate binding. Instead it seems to have a structural role on the positioning of the loop formed by the motif 2. Additional studies are necessary to tell which of the amino acids of the conserved region 2 can be implicated directly in substrate binding.

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