Aspartyl-tRNA synthetase from *Escherichia coli*: cloning and characterisation of the gene, homologies of its translated amino acid sequence with asparaginyl- and lysyl-tRNA synthetases

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ABSTRACT

By screening of an *Escherichia coli* plasmidic library using antibodies against aspartyl-tRNA synthetase (AspRS) several clones were obtained containing *aspS*, the gene coding for AspRS. We report here the nucleotide sequence of *aspS* and the corresponding primary structure of the aspartyl-tRNA synthetase, a protein of 590 amino acid residues with a Mr 65,913, a value in close agreement with that observed for the purified protein. Primer extension analysis of the *aspS* mRNA using reverse transcriptase located its 5′-end at 94 nucleotides upstream of the translation initiation AUG; nuclease S1 analysis located the 3′-end at 126 nucleotides downstream of the stop codon UGA. Comparison of the DNA-derived protein sequence with known aminoacyl-tRNA sequences revealed important homologies with asparaginyl- and lysyl-tRNA synthetases from *E.coli*; more than 25% of their amino acid residues are identical, the homologies being distributed preferencially in the first part and the carboxy-terminal end of the molecule. Mutagenesis directed towards a consensus tetrapeptide (Gly-Leu-Asp-Arg) and the carboxy-terminal end showed that both domains could be implicated in catalysis as well as in ATP binding.

INTRODUCTION

Aminoacyl-tRNA synthetases share a common function, which is to promote the attachement of an amino acid to its cognate tRNA in a highly specific two step reaction. Although they catalyse the same type of reaction, these enzymes show considerable variation in size, subunit structure and amino acid sequence (1, 2). At present, cristallographic data are available for two enzymes: MetRS from *E.coli* (3, 4) and TyrRS from *B.stearothermophilus* (5, 6) and for the complex GlnRS-$tRNA^{Gln}$ from *E.coli* (7). Moreover, SerRS from *E.coli* (8) and the complex AspRS-$tRNA^{Asp}$ from yeast (9, 10) are under investigation by X-ray diffraction. Until now, the only signature sequence, whose functional importance has been demonstrated, is the HIGH consensus tetrapeptide detected in 9 heterologous synthetases (11) and thoroughly investigated in the case of TyrRS from *B.stearothermophilus* (12–14). In other cases a KMSKS consensus sequence has been detected which should be localised in the vicinity of the CCA arm of the tRNA (15, 16). More extensive homologies are found between MetRS, ValRS, IleRS and LeuRS (17), and on the other hand between yeast AspRS, AsnRS and LysRS from *E.coli* (18, 19). The meaning of these homologies is very poorly understood although it obviously reflects the preservation of some functionally essential elements probably already present in a common ancestral molecule (17). The arrangement of functional domains in yeast AspRS was previously probed by the site-directed mutagenesis approach (20) which demonstrated the implication of the central part and the C-terminal end of the enzyme in its activity. A complementary approach to AspRS mutant engineering and their enzymatic analysis would consist in examining AspRS from other species for primary structure homologies. This led us to clone and sequence the AspRS gene from *E.coli*. Since no mutant was available to clone by the complementation method, we purified the protein to homogeneity, raised antibodies in rabbits and screened a plasmidic DNA library from *E.coli* by a radioimmunological procedure. We report here the cloning of *aspS* from *E.coli*, the primary structure of the protein and the mapping of the *aspS* mRNA termini. Finally the structure-activity relationship of AspRS was investigated by point mutations and progressive C-terminal end deletions of the protein; kinetic parameters variations were analysed and their significance discussed in terms of functional organisation.

MATERIALS AND METHODS

Enzymes and chemicals

Restriction endonucleases, T4 DNA ligase and polymerase, terminal deoxynucleotidyl transferase, lysozyme, *E.coli* DNA polymerase I, unfractionated *E.coli* tRNA were all from Boehringer Mannheim. Bacterial alkaline phosphatase and

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proteinase K were from Appligene. MLV reverse transcriptase and mung bean nuclease were from Bethesda Research Laboratories. Sequenase sequencing kits were purchased and used according to United States Biochemical Corporation. [^{14}C] L-aspartic acid was from the Commissariat à l'Energie Atomique (Saclay, France); [^{125}I] Protein A from Du Pont New England Nuclear and [α-^{35}S] dATP from Amersham. DEAE-cellulose was obtained from Whatman; DEAE-Sephacel, HA-Ultrogel, MonoQ from Pharmacia-LKB and Affigel 102 from Bio-Rad. Complete Freund adjuvant was from Difco Laboratories.

Bacterial strains, plasmids and bacteriophages

The *E.coli* strains used were: BJ5183 (F^-, recBC, sbcB, endA, galK, met, thi-1, bioT, res, mod+,str^r), JM103 (Δ(lac-proAB), thi-1, strA, supE, endA, sbcB15, hsdR4, F'(traD36, proAB$^+$, lacIqZΔM15)),TG1 (Δ (lac-proAB), supE, thi, hsdD5, F'(traD36,proAB,lacIqZ ΔM15)). Plasmids pBR322, and pBluescript KS were grown in *E.coli* strain BJ5183 and TG1. Bacteriophages M13mp18 and M13mp19 were grown in *E.coli* strain JM103.

Bacterial growth, DNA isolation and manipulation

Growth conditions for obtaining plasmidic DNA, double-stranded or single-stranded M13 DNAs were described by Maniatis *et al.* (21). Plasmids and replicative form of M13 were prepared according to (21). Single stranded phage DNA isolation was accomplished according to Amersham procedures. DNA manipulations, gel electrophoresis, recovery of DNA fragments and *E.coli* transformations were performed using standard procedures (21).

Aminoacylation assay

Aminoacylation activity of AspRS was determined by adding 10 μl of various protein dilutions to 90 μl of the following reaction mixture: 100 mM Tris HCl (pH 7.5), 30 mM KCl, 2 mM ATP, 10 mM $MgCl_2$, 1 mM DTT, 2 mg/ml of unfractionated *E.coli* tRNA, 0.1 mg/l bovine serum albumin and 0.1 mM [^{14}C] aspartic acid (25,000 dpm/nmol). At various time intervals of incubation at 37°C, 20 μl of the reaction mixture were assayed for [^{14}C] aspartyl-tRNA^{Asp} by the method described previously (20) and the initial rates were determined in each case.

The Km values for aspartic acid, ATP and tRNA^{Asp} were determined under the conditions described above, except that the concentrations of the tested substrates varied: aspartic acid (0.0125−0.1 mM), ATP (0.0375−1.5 mM), total tRNA (16−100 μM); total tRNA contained about 2.5% tRNA^{Asp}. In this last case, [^{14}C] aspartic acid (50,000 dpm/nmol) was used. For Km measurements an excess of Mg^{2+} over ATP was maintained constant at 20 mM.

ATP/PPi exchange assay

The standard reaction mixture contained 100 mM Hepes (pH 7.5), 8 mM $MgCl_2$, 2 mM [^{32}P]PPi, 2 mM ATP, 5mM aspartic acid, 10 mM KF and 1−350 μg protein/ml. After various incubation times at 37°C the [^{32}P]ATP synthesised was determined in aliquots from the incubation mixture. In each case, initial velocities were determined.

Purification of Aspartyl-tRNA synthetase

All steps were carried out at 4°C. *E.coli* MRE600 cell paste (400g) was suspended in 1000 ml of buffer A (100 mM Tris HCl (pH 8), 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT). The cell suspension was disrupted in a continuous glass beads grinder (Dyno-Mill, Willey, Switzerland). Cell debris were removed by centrifugation at 14,000×g for 90 min. The supernatant (1000 ml) was adjusted to pH 7.8 by addition of ammonium hydroxide and subjected to ammonium sulphate fractionation. 250 g ammonium sulphate were added under stirring over a period of 30 min and insoluble material was removed by centrifugation at 14,000×g for 45 min. Additional 160 g ammonium sulphate were added to the supernatant fraction and centrifugation was repeated for 45 min. The pellet was suspended in 150 ml of buffer B (20 mM potassium phosphate (pH 7.5), 1 mM EDTA, 10% v/v glycerol) dialysed twice for 8-hours against 10 litres of the same buffer. The dialysed protein solution was then centrifuged for 30 min at 19,000×g and the supernatant applied at 180 ml/h on a 7×190 cm DEAE-cellulose column equilibrated in buffer B. The elution was done with a 6000 ml linear gradient from buffer B to buffer C (250 mM potassium phosphate (pH 6.5), 1 mM EDTA, 10% v/v glycerol). Fractions containing aminoacylation activity were dialysed against buffer D (10 mM potassium phosphate (pH 7.5), 1 mM EDTA, 10% v/v glycerol) and loaded at 160 ml/h onto a 5.5×12.5 cm HA-Ultrogel column. The column was developed with a linear gradient (3000ml) from buffer D to buffer E (50 mM potassium phosphate (pH 7.5), 1 mM EDTA, 10% v/v glycerol). Active fractions were applied at 90 ml/h on a 2.5×20 cm column of Affigel 102 in buffer F (1 mM potassium phosphate (pH 7.5), 1 mM EDTA, 10% v/v glycerol). The elution was performed with a linear gradient (1000 ml) from buffer F to buffer E. Final purification was performed with a FPLC column Mono Q HR 5/5: the Affigel 102 fraction was concentrated, dialysed against buffer G (10 mM Tris HCl (pH 7.5)) and loaded onto a 1 ml Mono Q column equilibrated in buffer G. Elution was done at 1 ml/min with a gradient of KCl from 0 to 700 mM in buffer G. The fraction corresponding to AspRS activity (eluted at 200 mM KCl) contained a pure protein as could be shown by SDS-PAGE.

Antibodies preparation

Rabbits were immunised at 15-day intervals by repeated subcutaneous injections of 100 μg of enzyme dissolved in 500 μl 10 mM potassium phosphate (pH 7.5), 150 mM NaCl, and emulsified in 500 μl of complete Freund's adjuvant. One week after the last injection, rabbits were bled and serum, adjusted to 50% glycerol, was conserved at −20°C. The specific antibodies exhibit a ten ng sensitivity towards AspRS.

N-Terminal sequence analysis

Homogeneous aspartyl-tRNA synthetase (about 2 nmoles in 100 mM N-methyl morpholine buffer (pH 8)) was used for N-terminal sequence analysis by automated Edman degradation on an Applied Biosystems 470A gas-liquid phase protein sequencer. The N-phenylthiohydantoin (PTH) aminoacids were separated and identified by an on-line PTH analyser 120A (Applied Biosystems).

Determination of the molecular weight of aspartyl-tRNA synthetase

Molecular weights of AspRS and mutant derivatives were determined by gel filtration on a 60-cm long TSK SW 3000 column (Beckmann). Elution was performed at a flow rate of 0.4 ml.min^{-1} with a buffer 10 mM potassium phosphate (pH

Table I: Purification of aspartyl-tRNA synthetase from *E.coli*

Purification step	Total protein (mg)	Activity (µmole/min)	Specific Activity (µmole/min/mg)	Enrichment (Fold)	Yield (%)
Crude extract	33,300	383,000	11.5		100
Ammonium sulphate fraction	14,000	254,800	18.2	1.6	66.5
DEAE-cellulose fraction	670	123,300	184	16	32.2
HA-Ultrogel fraction	60	61,800	1030	90	16.1
Affigel 102 fraction	12.6	34,900	2770	240	9.1
Mono Q - Pure enzyme	3.5	21,000	6000	522	5.5

7.5), 150 mM NaCl. The column was calibrated with standard proteins (Boehringer Mannheim).

Radioimmunological screening

BJ5183 was transformed with an *E.coli* genomic library using the $CaCl_2$ procedure. The transformation mixture was incubated overnight at 37°C on LB-Ampicillin. 3.10^3 transformants were obtained per plate (Ø = 86 mm). Once a replica of the clones was done, the cells were lysed by chloroform vapours. After a 10 min period, residual vapours were removed under a hood and the clones covered with 4 ml LB top agar. A dry nitrocellulose filter was overlaid and the plates were incubated at 4°C for 14-hours. Then, the filters were removed and treated by the antibody-[^{125}I]protein A procedure described before (22). Dried filters were exposed at −80°C for 14-hours in the presence of XAR5 Kodak film and intensifying screen. Positive clones were purified by two additional screening cycles.

Preparation of crude extracts

E.coli strains were grown in 100 ml of LB Amp (100 µg/ml) at 37°C to stationary phase. The cells were harvested by centrifugation at 12,000×g, washed with bidistilled water and then resuspended in the lysis buffer (100 mM Tris HCl (pH 8), 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT). This mixture was transferred into a 1.5 ml Eppendorf tube and submitted to sonication for 4×20 seconds at 45 V with an Ultrasons-Annemasse apparatus (type 250TS20K). Cell debris were eliminated by centrifugation at 12,000×g and the supernatant collected. Protein concentration was estimated by the optical method of Ehresmann *et al.*(23).

Immunological titration of aspartyl-tRNA synthetase in the crude extracts

To titrate the AspRS content, the crude extracts were diluted to 5 µg of protein/ml in 100 mM Tris HCl (pH7.5), 30 mM KCl, 0.1 mg/ml bovine serum albumin. To a constant amount of protein (50−700 ng, depending on the crude extract) increasing amounts of antibodies were added. The mixtures were adjusted to 25 µl with the above buffer and incubated at 0°C for 15 min. The remaining aminoacylation activity was determined at 37°C after addition of 10 µl of the incubation mix to 90 µl of the complete aminoacylation reaction mixture. The concentration of AspRS in the crude extract could be established using a standard inactivation curve performed in the same conditions with pure AspRS.

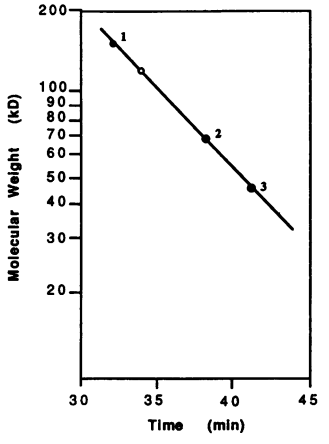

Figure 1. Determination of the molecular weight of AspRS from *E.coli* under non denaturing conditions by chromatography on a TSK SW3000 column. The standard proteins used and their molecular weight ($\times 10^3$) were: 1, aldolase (158); 2, bovine serum albumin (68); 3, ovalbumine (45). The elution position of AspRS is indicated by an open circle.

Analysis of AspRS-specific proteins by immunoblotting

Protein samples were run on 10% polyacrylamide gels in the presence of 0.1% sodium dodecylsulphate according to Laemmli (24). Proteins were electrophoretically transferred to nitrocellulose paper, treated with rabbit antiserum and [^{125}I] protein A as described (22).

DNA sequencing

DNA from pAspS1 was subjected to restriction digestion and the resulting 1.1 kbp *Pst*I-*Eco*RI and 2.75 kbp *Pst*I-*Hind*III fragments were purified by the standard procedures; subcloning of these fragments was performed in M13mp18 and M13mp19. Their complete DNA sequence was determined by the dideoxy-DNA sequencing method of Tabor and Richardson (25) on a set of overlapping subclones generated by the procedure of Dale *et al.* (26). DNA and protein sequences were analysed by using the programmes of the University of Wisconsin Genetics Computer Group (27).

Preparation of RNA

The AspRS overproducing strain containing pAspS1 was grown exponentially in 20 ml LB Amp (100 µg/ml) until OD_{700} = 0.5

was reached. Cells were harvested by centrifugation at 4000×g, washed twice with bidistilled water and resuspended in 10 ml 100 mM sodium acetate (pH 5.1), 1 mM EDTA. Five ml of TE-saturated phenol was added and cells were lysed by a ten-min period of vigorous shaking. After centrifugation, the aqueous phase was re-extracted by phenol, ethanol-precipitated and washed by a large volume of 80% ethanol.

Primer extension analysis and S1 nuclease mapping

For primer extension analysis (28), a 25-mer oligonucleotide (5′-GACCCAACCACACAGAGTCACCTGC) complementary to the AspRS mRNA sequence near the translation initiation codon was synthesized on a Applied Biosystems 381A apparatus and 5′-labelled with [γ-^{32}P] ATP using T4 polynucleotide kinase. The primer (4.10^4 dpm) and 100 μg of total cellular RNA were dried together, resuspended in 100 μl 80% formamide, 100 mM Pipes (pH 6.4), 400 mM NaCl, 1 mM EDTA, heated 5 min at 70°C and incubated overnight at 42°C. The hybridization mix was ethanol precipitated and the pellet resuspended in 50 mM Tris HCl (pH 8.3), 6 mM $MgCl_2$, 40 mM KCl, 10mM DTT, 1 mM dNTP ; then, 200 units of Moloney Murine Leukaemia Virus reverse transcriptase were added and extension was allowed to proceed at 37°C for 30 min. Following ethanol precipitation, the primer extension products were fractionated by electrophoresis on a 6% sequencing polyacrylamide gel in parallel to a Sanger sequencing reaction mix generated with the same primer (29).

The S1 nuclease method used to identify the 3′ end of the *aspS* transcript is derived from the procedure of Berk and Sharp (30). A 411-bp *Hinf*I restriction fragment containing the 3′ noncoding region of *aspS* extending from position 1777 to position 2188 was labelled at its 3′ end with [α-^{32}P] ddATP (5000 Ci/mmole) using the Klenow fragment of the DNA polymerase I. After *Bst*EII digestion and electrophoretic fractionation on a 5% polyacrylamide gel, a 227-bp *Hinf*I−*Bst*EII labelled probe was obtained. Total cellular RNA (100 μg) and 5.10^4 dpm of the [^{32}P]-labelled restriction fragment were hybridised together in 40 μl of buffer (80% formamide, 50mM Pipes (pH 6.5) and 400 mM NaCl), denatured at 85°C for 10-min, incubated at 42°C for 16-hours and placed on ice. 260 μl of cold Mung Bean Nuclease buffer (30 mM sodium acetate (pH 5.0), 100 mM NaCl, 2 mM $ZnCl_2$, 10% glycerol) and 25 units of Mung Bean Nuclease were added. After digestion at 37°C for one hour, the reaction was stopped by phenol-chlorophorm extraction. The protected DNA fragments were precipitated by ethanol and fractionated on a 6% polyacrylamide sequencing gel in parallel with a Maxam and Gilbert sequencing reaction mix (31).

Mutagenesis

Mutagenic oligonucleotides were assembled on an Applied Biosystems 381A DNA synthesiser. The synthetic oligonucleotides used for carboxy-terminal deletions were the following: −15 residues, 5′-GCTAACCCGACTGCATG-ATATGACTC; −30 residues, 5′-CCACGGCGGCATG-ATATGACTC; −45 residues, 5′-GCTGACCGGCACCTGAT-ATGACTC. Point mutation T 1604 → C was performed with: 5′-GCATTCGGTCCTGACCGTCTG. The DNA template used

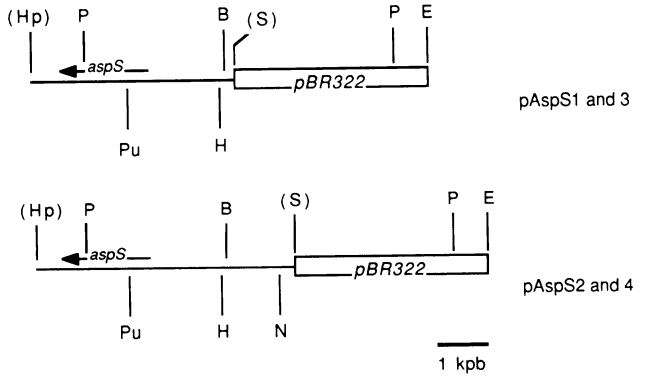

Figure 2. Restriction endonuclease maps of pAspS1−4. The open bars represent vector pBR322. The inserts are indicated by thin lines; the position of the protein-coding sequence is indicated by a black arrow. The abbreviations used are: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*II; N, *Nsi*I; P, *Pst*I; Pu, *Pvu*II; S, *Sau*3A.

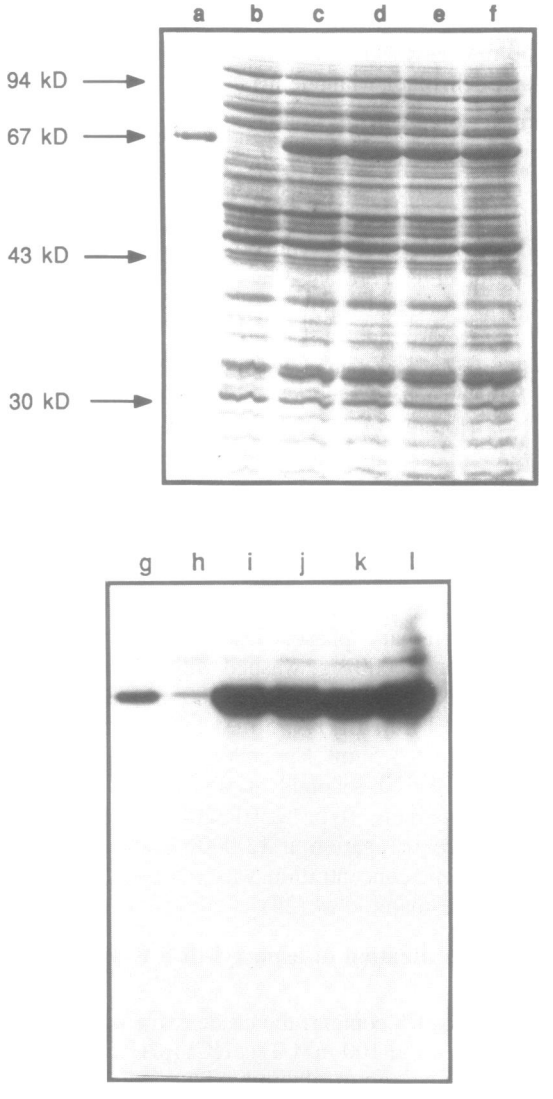

Figure 3. Characterisation of *aspS* expression products in *E.coli* harbouring pAspS1−4. Crude extracts were prepared by sonication of cells from stationary phase cultures, separated by SDS-10%-PAGE and stained with Coomassie blue (lanes a−f) or subjected to Western blotting using antibodies and [^{125}I]protein A (lanes g−l). Lane a: 1μg of pure AspRS; lane b: 50 μg of the crude extract from the recipient BJ5183; lanes c−f: 50μg of crude extracts from BJ5183 transformed by pAspS1−4 respectively; lane g: 10ng of pure AspRS; lane h: 10μg of crude extract from the recipient BJ5183; lanes i−l: 10 μg of crude extracts from BJ5183 harbouring pAspS1−4.

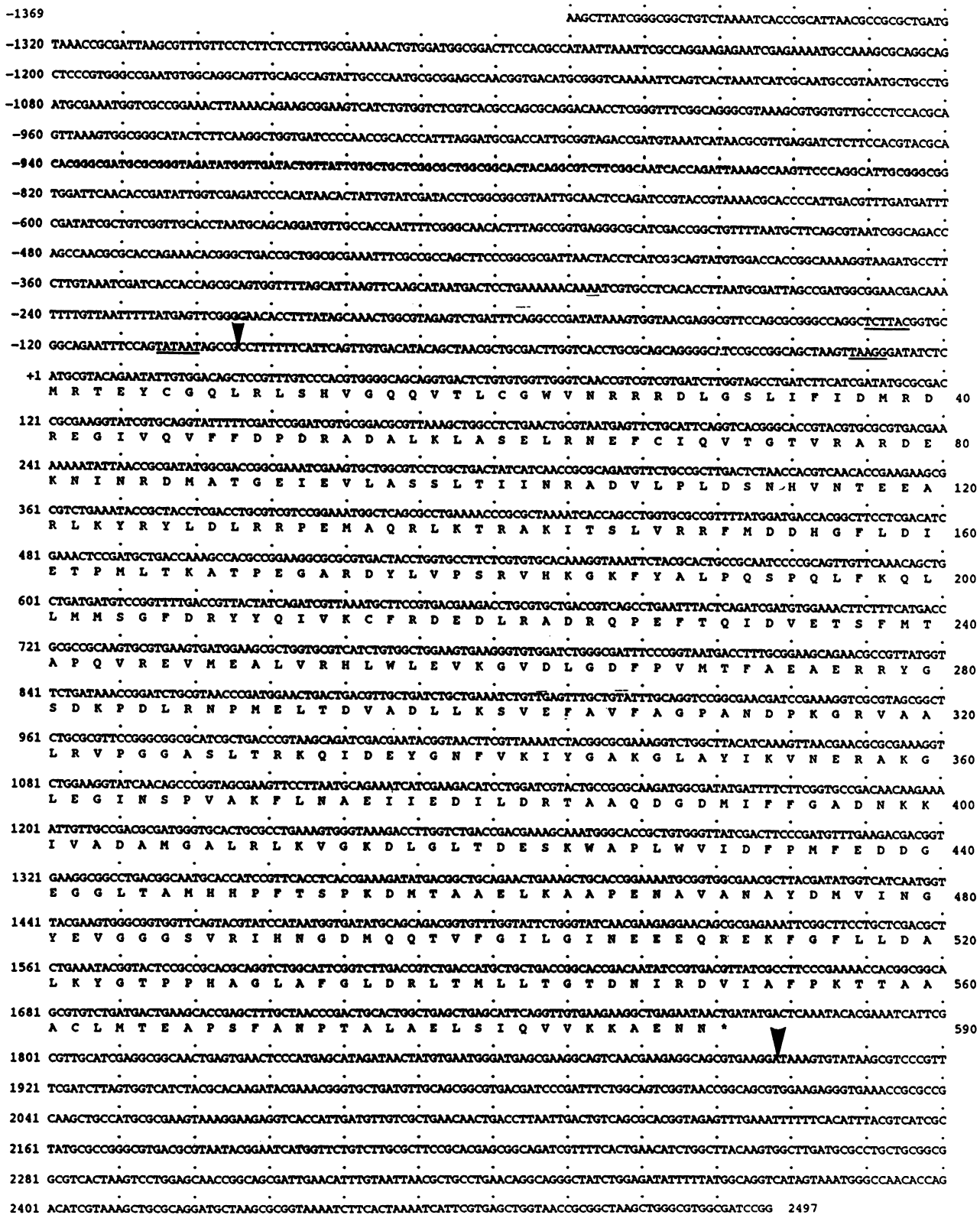

Figure 4. Primary structure of *aspS*, its flanking regions and the corresponding amino acid sequence. Nucleotides are numbered beginning with +1 at the first nucleotide of the AUG translation initiation triplet. Amino acids are numbered beginning with 1 at the initiator methionine. Underlining shows possible regulatory sequences like −35 and −10 transcription control elements and the ribosome binding site. The 5′ and 3′-end of the *aspS* transcript are marked with black arrows; the position of the 5′-end was corrected for a −1 nucleotide value with regard to figure 5 to account for the [^{32}P] phosphate used for labelling the oligonucleotide.

for mutagenesis was pBluescript KS- containing the 3.45 kbp *Eco*RI-*Hind*III restriction fragment carrying the *aspS* gene. Packaged single stranded DNA was generated by superinfecting the transformants with the helper phage M13K07 according to Stratagene procedures. *In vitro* mutagenesis reactions were carried out using a kit from Amersham; the synthesised double

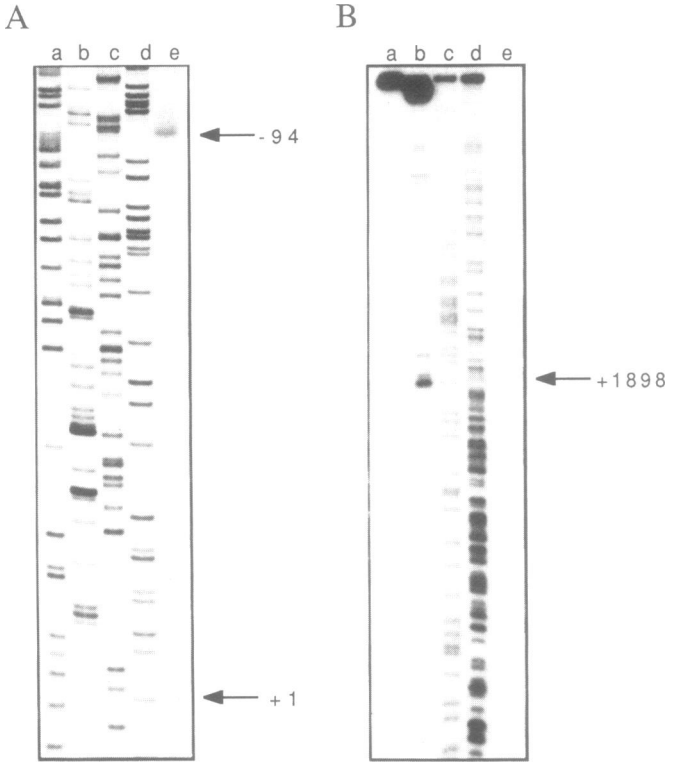

Figure 5. Determination of the termini of the *aspS* transcription product. A: Mapping of the 5′-terminus of *aspS* mRNA. Total RNA (100μg) was prepared as described in Materials and Methods and hybridised to 4.10^4 dpm of [^{32}P]phosphorylated primer complementary to position 48 to 72 in figure 4. After ethanol precipitation and MLV-reverse transcriptase extension, the products were fractionated on a 6% PA-urea gel. Lanes a−d: dideoxy-sequencing pattern of *aspS* in the presence of the primer (non-phosphorylated) used for 5′-mapping (ACGT respectively); lane e: primer extension of total RNA with [^{32}P]phosphorylated primer. The numbers refer to positions in the DNA ladder. B: S1 nuclease mapping of the 3′ terminus of the *aspS* mRNA. A 3′-end labelled *HinfI-BstEII* fragment (position 1777−2004 in figure 4) was heat denatured, hybridized to total RNA (100μg) and treated with S1nuclease (see Materials and Methods). The nuclease S1 protected fragments were fractionated on a 6% PA sequencing gel. Lane a: 227bp-*HinfI-BstEII* fragment [^{32}-P]-labelled at the *HinfI* site used as DNA probe; lane b: DNA-RNA hybrids formed at 42°C, after nuclease S1 digestion (the major protected end is indicated by an arrow; residual probe, without the 5 protruding nucleotides of the *BstEII* site is present behind the excess of the 227bp DNA fragment); lanes c and d: Maxam and Gilbert sequencing reaction of the DNA probe (C+T and A+G respectively); lane e: DNA probe in absence of RNA and after treatment with S1 nuclease. By determination of the mRNA 3′ end (position 1898), we took into account the fact that the indicated base in the ladder sequence is cleaved off during the Maxam and Gilbert reaction.

stranded thio-DNA was used to transform *E.coli* TG1. Clones carrying mutant phagemids were identified by DNA sequencing of the double stranded DNA obtained by the small scale alkaline preparation method (32).

RESULTS AND DISCUSSION

Purification of AspRS from *E.coli*

Aspartyl-tRNA synthetase from *E.coli* was purified by ammonium sulphate precipitation, chromatography on DEAE-cellulose, HA-ultrogel, Affigel 102, and Mono Q as described in Experimental Procedures. 3.5 mg of pure enzyme were isolated from 400 g of wet cells. The enzyme was purified to apparent homogeneity after a 522-fold enrichment and a yield of 5.5% was obtained (Table I). As shown in Fig.1, *E.coli* aspartyl-tRNA synthetase like the yeast enzyme (33) exhibits an α_2 oligomeric

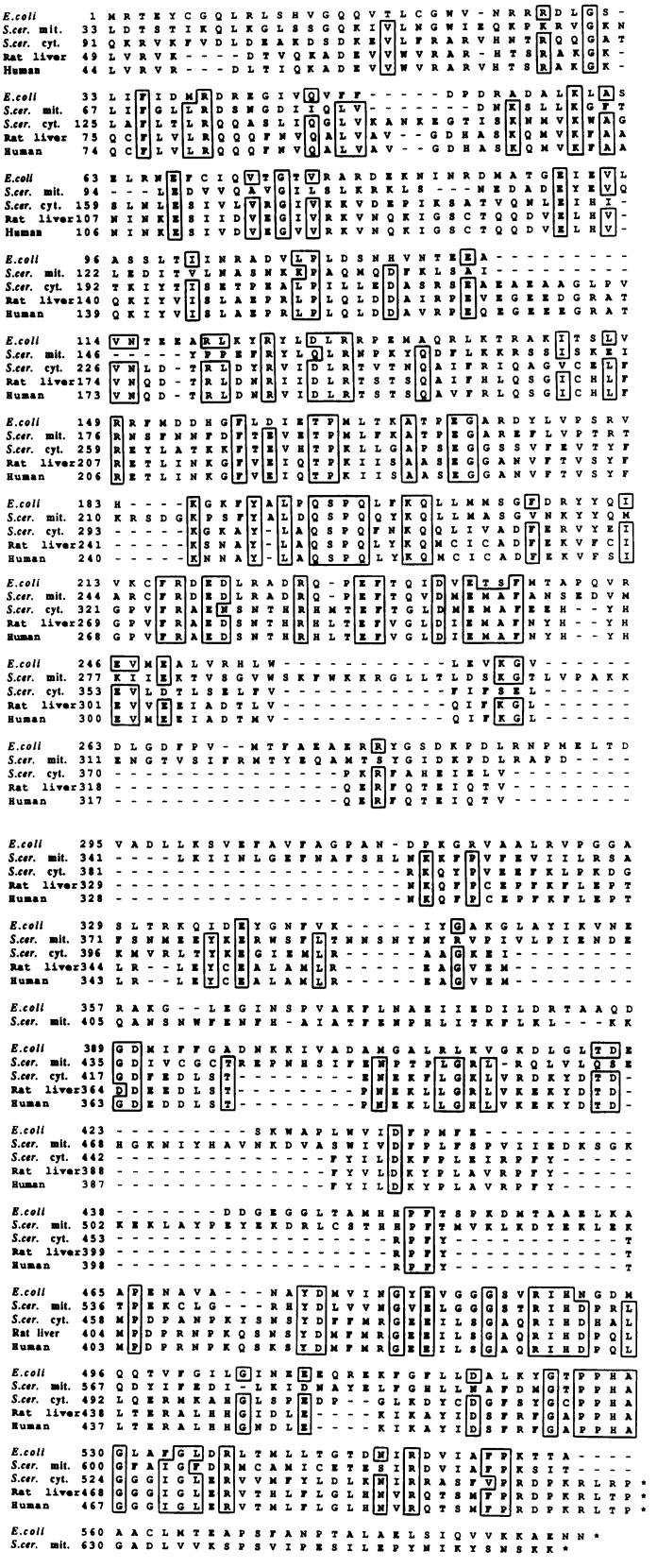

Figure 6. Sequence alignment of aspartyl-tRNA synthetases from different origins. The sequence of the *E.coli* enzyme is aligned with the primary structures of yeast mitochondrial (39), yeast cytoplasmic (38), rat liver (40) and human (41) enzymes. Identical amino acid residues are boxed if four or more enzymes have a common residue at the same alignment position. Numbers refer to amino acid position.

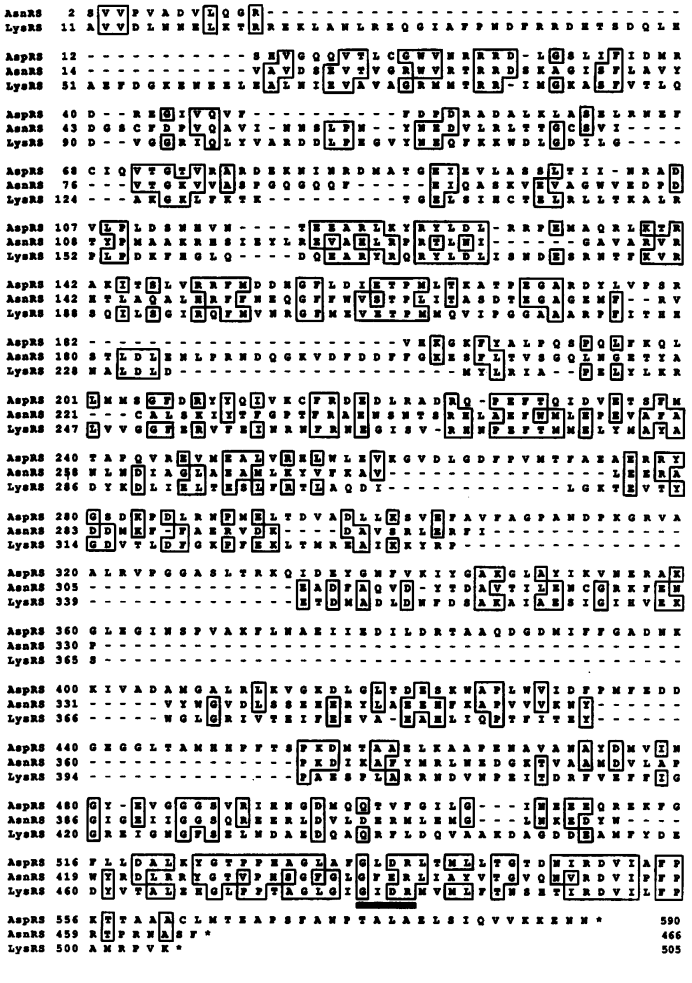

Figure 7. Sequence alignment of the aspartyl-, asparaginyl-(18), and lysyl-tRNA synthetase (encoded by *lysS*)(19) of *E.coli*. Identical amino acid residues are boxed if two or more sequences have a common residue at the same alignment position. Ten regions of substantial homology, interrupted by variable peptide extensions, are found between the 3 enzymes. The position of the tetrapeptide Gly-Leu-Asp-Arg is underlined by a heavy line.

structure with a subunit Mr of 65,000 as established by polyacrylamide gel electrophoresis under denaturing conditions. This result is consistent with the previous finding of Åkesson and Lundvik (34).

Cloning of the *E.coli* aspartyl-tRNA synthetase gene

The *E.coli* aspartyl-tRNA synthetase gene was isolated from a plasmid pool containing partial digested *Sau*3A−*Hpa*II fragments of *E.coli* chromosomal DNA inserted in the *Bam*HI-*Cla*I sites of pBR322 (35). The gene was selected in the *E.coli* BJ5183 host strain using a radioimmunological screening procedure: transformant *E.coli* cells harbouring the AspRS gene were expected to overproduce AspRS protein and by consequence to display a stronger signal in the presence of antibodies than the strain producing only the genomically encoded enzyme. Among the 20.000 Amp^r transformants obtained, four clones were purified after three radioimmunological screening cycles: they showed a strong response to antibodies-[^{125}I]protein A treatment. The plasmids isolated from these putative AspRS expressing transformants were designated pAspS1, pAspS3 (8.15 kbp) and pAspS2, pAspS4 (9.4 kbp)(Fig.2). Their crude extracts showed a 30-fold as high aspartyl-tRNA synthetase activity compared to the wild type strain; thus, the enzyme expressed

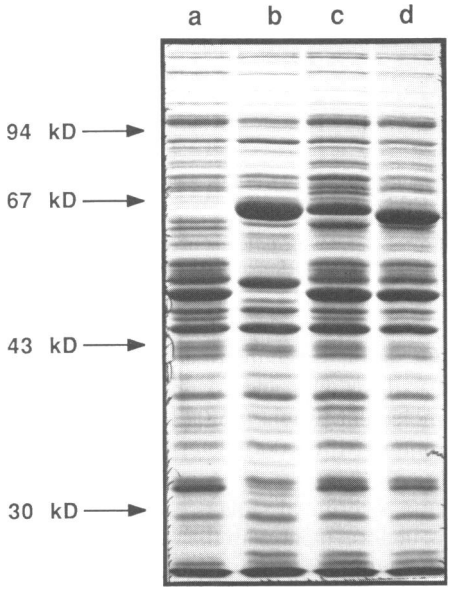

Figure 8. Fractionation by SDS-10%-PAGE of crude extracts from control and transformed *E.coli* TG1. 50μg of lysates from the different clones were analysed: from the recipient TG1 (lane a), from the transformants harbouring pBluescript-*aspS* (lane b), pBluescript-*aspS*-LP535 (lane c) and pBluescript-*aspS*-Δ_C15 (lane d). It has to be noticed that the fractionation pattern corresponding to the native enzyme crude extract slightly differs from the others by the absence or variation in intensity of some protein bands; this may be explained by a regulatory effect on these proteins, induced by the high amount of AspRS (the mutant proteins in lanes c and d being less expressed or less active).

in *E.coli*−pAspS1 to 4 cells reached about 5% of the total soluble protein amount as could be calculated taking into account the specific activity of the purified enzyme. In order to check whether the activity was associated with a full-length protein in the overproducing strains, crude extracts were analysed by the Western blot procedure after electrophoretic separation on a sodium dodecyl sulfate-polyacrylamide gel and transfer to nitrocellulose. AspRS was detected using the specific antiserum and [^{125}I]-labelled protein A. As shown in Fig.3, a protein which co-migrates with the purified aspartyl-tRNA synthetase (lane g) is present in the crude extract of the recipient strain (lane h); the amount of this protein is strongly increased in the strains harbouring pAspS1 and pAspS2 (lanes i-l).

Determination of the nucleotide sequence of the *aspS* gene of *E.coli*

Digestion of pAspS1 with *Eco*RI, *Pst*I and *Hind*III restriction endonucleases yielded two fragments of 2.75 and 1.1 kbp in addition to the 3.7 kbp and 0.75 kbp fragments originating from pBR322. Subcloning of *aspS* gene fragments was performed in bacteriophages M13mp18/19. DNA sequencing of the two fragments, which together contain the entire *aspS* gene, was performed on both strands by sequencing a set of overlapping M13 clones obtained by limited T4 DNA polymerase digestion according to (26); the truncated inserts were sequenced using modified T7 DNA polymerase. The sequencing of the internal *Pst*I region was carried out directly on pAspS1 DNA using an adequate synthesised oligonucleotide (5′-GCACCATCCGTTCA-CCT). This demonstrates definitely that this region does not contain two adjacent *Pst*I restriction sites.

A long open reading frame of 1770 nucleotides was found on one strand (position +1 to +1770 in Fig. 4). The deduced amino

Table 2. Catalytic parameters of mutated aspartyl-tRNA synthetases

Protein encoded by	Intracellular content (%)	Specific activity exchange (nmol.mg^{-1}.min^{-1})	Specific activity acylation (nmol.mg^{-1}.min^{-1})	Km in the acylation reaction for Asp (μM)	for ATP (μM)	for tRNA^{Asp} (μM)	Oligomeric structure
pBluescript-*aspS*	21	8950	4250	60	60	0.55	α_2
pBluescript-*aspS*-Δ_C15	17	4440	3350	100	364	1.3	α_2
pBluescript-*aspS*-LP535	4.1	3550	1085	110	1190	1	α_2

The proteins expressed from the different plasmids are the following: pBluescript-*aspS*: native AspRS; pBluescript-*aspS*-Δ_C15: AspRS deleted of the last 15 residues; pBluescript-*aspS*-LP535: AspRS mutated at position 535 (L→P).

acid sequence starting from the first in-phase Met codon includes 590 amino acid residues and corresponds to a protein, whose calculated molecular weight (65,913) is in good agreement with the value (subunit Mr 65,000) determined by SDS-PAGE of the protein (Fig. 3).

The enzyme N-terminal sequence, as deduced from the DNA sequence, is in agreement with the 11 first N-terminus residues identified by automated Edman degradation of the purified AspRS: Met-Arg-Thr-Glu-Tyr-Cys-Gly-Gln-Leu-Arg-Leu. This result confirms unambiguously that the DNA insert corresponds to the *aspS* gene.

Codon Usage

We compared the codon usage frequency of aspartyl-tRNA synthetase with the average frequency of codon usage defined by Konigsberg and Godson (36). We found that AspRS shows a pattern of non random codon usage very similar to the one of highly expressed genes from *E.coli* except for codon GUG (Val) which is preferred to GUU and codon UGU (Cys) which is used preferentially to UGC.

Extinction coefficient of aspartyl-tRNA synthetase from *E.coli*

The extinction coefficient of AspRS at 280 nm, calculated on the basis of the amino acid composition deduced from the DNA sequence is 0,55 mg^{-1}.ml^{-1}.cm^{-1} when assuming an extinction contribution of 5480 mole^{-1}.cm^{-1} for tryptrophan, 1180 mole^{-1}.cm^{-1} for tyrosine (37) and a Mr of 65,913. This value is close to the extinction coefficient of 0.5 mg^{-1}.ml^{-1}.cm^{-1} determined directly from the absorption at 280 nm of a known concentration of protein as determined by nitrogen content.

Termini of *aspS* mRNA and consensus elements

The initiation site of transcription was determined by primer extension using reverse transcriptase and a 5′ end labelled synthetic oligonucleotide (see Materials and Methods). Extension products were separated on a polyacrylamide sequencing gel in parallel with a dideoxy-sequencing reaction mix of *aspS* obtained with the same oligonucleotide primer. Figure 5A shows a major signal at the level of position −94 of the ladder; taking into account the presence of 5′ end phosphate on the synthetic oligonucleotide, this indicates that the mRNA start is located 95 nucleotides upstream of the initiation codon. At positions −100 and −126 we found the canonical 'TATA' and 'TTGAC like' sequences, which are suspected to be transcription control elements (38). At position −9 there is a UAAGG sequence which is complementary to the 3′ end of the 16S rRNA and thus probably corresponds to a ribosome binding site (39).

The region downstream of the *aspS* stop codon was analysed by S1nuclease mapping to determine the transcription termination site. The experiment was performed with an excess of 3′ labelled double stranded DNA probe (*Hinf*I / *Bst*EII, position +1777 to +2004 in Fig. 4) and total mRNA synthesised in the overproducing strain carrying pAspS1. A major protected fragment corresponding to the transcription termination site was mapped at 125 nucleotides downstream of the stop codon UGA. No structure of dyad symmetry followed by an U-rich sequence was detected in the neighbourhood suggesting that transcription termination of the *aspS* mRNA is not a rho-independent event.

Primary structure homologies between aspartyl-tRNA synthetase and other aminoacyl-tRNA synthetases

Using UWgcg computer programs (27), we compared the amino acid sequence of AspRS from *E.coli* with the primary structures of the 18 other known aminoacyl-tRNA synthetases from *E.coli* and with mitochondrial and cytoplasmic AspRS from *S. cerevisiae* (40, 41), rat liver (42) and human (43). As expected, the strongest overall homology was observed when comparing the primary structures of the AspRS from different organisms. The highest homology was found between the bacterial and the yeast mitochondrial enzymes where 36.5% of the residues are identical; these two enzymes have about 26−29% residues identical to those of other, eukaryotic AspRS. Figure 6 shows an alignment of the five known AspRS primary structures. Large extensions are found at the N-terminal end of the eukaryotic enzymes, the largest of them (about 90 residues long), is present in the cytoplasmic yeast AspRS. These extensions are suspected to play a role in the compartmentalisation of these molecules inside the cell (44) by interactions with cytoplasmic components not yet identified (44) or to participate in the formation of the multienzymatic complexes as observed in higher eukaryotes (45). Extensive homologies between the 5 enzymes are essentially found in the first half of the molecules and the carboxy-terminal regions. Large peptide extensions are present in the bacterial and mitochondrial yeast enzymes.

Futhermore, as expected from recent observations (18, 19), we found homologies between AspRS, LysRS (19) and AsnRS (18) from *E.coli*. Figure 7 shows an alignment of these three bacterial enzymes: identical residues are boxed, underlining stretches of strong homologies in the first part of the molecules and at the carboxy-terminal end where these homologies are the most pronounced around a tetrapeptide Gly-Leu-Asp-Arg previously described by Molina *et al.* (43) as being part of a potential ATP binding site. This tetrapeptide, was detected on the basis of its homology with an *E.coli* AlaRS peptide segment, which, as was suggested, is located in the ATP binding domain (46).

Mutagenesis of aspartyl-tRNA synthetase at its carboxy terminal end

The function of the carboxy-terminal end of *E.coli* AspRS was tested by the mutagenesis approach; four mutations were

constructed by oligonucleotide site-directed mutagenesis of the *aspS* gene as described in Materials and Methods. Three of them corresponded to deletions of 15, 30 and 45 amino acids from the carboxy-terminal end of the molecule, the fourth (named pBluescript-aspS-LR535) was a single base change at position 1604 leading to the substitution of Leu 535 by a Pro in the Gly-Leu-Asp-Arg consensus sequence. The effects of these mutations on enzyme properties were determined directly in crude extracts. Expression of the *aspS* gene in pBluescript was expected to be as efficient as in pAspS1 since the construction maintained the gene under control of its own promotor.

Deletion mutagenesis. Analysis by SDS-PAGE of crude extract corresponding to mutant enzymes revealed that deletion of the 30 and 45 last residues drastically affects the expression level of AspRS. No protein could be detected either on SDS-PAGE stained with Coomassie Blue or by Western blot analysis (data not shown). It may be suspected that these two mutations desorganise the molecules in such a way to render them presumably more susceptible to proteolysis than the full-length product. On the opposite, the mutant deleted for the 15 last residues (named pBluescript-*aspS*-Δ_C15) is very well overexpressed in *E.coli* (Fig.8) and reaches 17% of the cytoplasmic protein content as determined by immunological titration. Table II, which summarises the kinetic data measured for the mutated enzyme, shows that the truncated form exhibits decreased Vmax for the exchange reaction (by a factor of 2) and a significant increase of the Km value for ATP; the Km value for $tRNA^{Asp}$ is increased by a factor 2 whereas the oligomeric structure of the protein remains unaffected by the deletion as shown by gel filtration.

Point mutation mutagenesis. A single point mutation (Leu→Pro) at position 535 (named pBluescript-*aspS*-LP535) of AspRS affects its expression level as shown by SDS-PAGE of the mutant crude extract: a value of 4.1% relative to total cytoplasmic protein was obtained for the mutated protein instead of the 21% for the wild type protein (Fig. 8). This mutation, present in the motif Gly-Leu-Asp-Arg produces strong effects on the enzyme properties: it lowers exchange and acylation Vmax (by a factor of 2.5 and 4 respectively) and increases 20-fold the Km value for ATP. No effects were observed on the Km values for aspartic acid as well as on the oligomerisation capacity of the enzyme, whereas a two fold increase of the Km value for $tRNA^{Asp}$ was observed.

The kinetic data measured for these two types of mutant enzymes clearly indicate that the carboxy-terminal region of AspRS is involved either directly or through distant effects in ATP binding. Lowering of ATP binding may result in lower binding energy of the intermediate complex and this could account for the drastic diminution of the reaction rates; another hypothesis explaining this inactivation effect which may not be excluded, is that the catalytic site of AspRS would be stabilised by its C-terminal domain; mutation in this part of the molecule would lead to structural desorganisation and therefore inactivation of the catalytic site conformation.

In a present study we are now analysing by more conservative substitutions the role of the different residues of the Gly-Leu-Asp-Arg motif and the surrounding positions to get clearer informations concerning its function in catalysis.

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