Glutamyl-tRNA Synthetases of Bacillus subtilis 168T and of Bacillus stearothermophilus

CLONING AND SEQUENCING OF THE gltX GENES AND COMPARISON WITH OTHER AMINOACYL-tRNA SYNTHETASES*

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The glutamyl-tRNA synthetase (GluRS) of Bacillus subtilis 168T aminoacylates with glutamate its homologous tRNA^{Glu} and tRNA^{Gln} in vivo and Escherichia coli tRNA^{Glu} in vitro (Lapointe, J., Duplain, L., and Proulx, M. (1986) J. Bacteriol. 165, 88–93). The gltX gene encoding this enzyme was cloned and sequenced. It encodes a protein of 483 amino acids with a $M_\text{r}$ of 55,671. Alignment of the amino acid sequences of four bacterial GluRSs (from B. subtilis, Bacillus stearothermophilus, E. coli, and Rhizobium meliloti) gives 20% identity and reveals the presence of several short highly conserved motifs in the first two thirds of these proteins. Conserved motifs are found at corresponding positions in several other aminoacyl-tRNA synthetases. The only sequence similarity between the GluRSs of these Bacillus species and the E. coli glutamyl-tRNA synthetase (GlnRS), which has no counterpart in the E. coli GluRS, is in a segment of 30 amino acids in the last third of these synthetases. In the three-dimensional structure of the E. coli tRNA^{Glu}–GlnRS–ATP complex, this conserved peptide is near the anticodon region of their tRNA substrates. This region is involved in the specific interactions between these enzymes and the anticodon regions of their tRNA substrates.

The covalent attachment of amino acids to their cognate tRNAs catalyzed by the aminoacyl-tRNA synthetases (aaRSs) is one of the crucial steps in the accuracy of protein biosynthesis. Bacterial cells generally contain one aaRS for each amino acid species incorporated into proteins (Grüenberg-Manago, 1987); reported exceptions are the presence of two

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§ The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; GluRS, glutamyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase; TyrRS, tyrosyl-tRNA synthetase; kbp, kilobase pair(s).

lsyl-tRNA synthetases in Escherichia coli (Irlshfield et al., 1981), of two threonyl-tRNA synthetases in Bacillus subtilis (Putzer et al., 1990), and the absence of a glutaminyl-tRNA synthetase (GlnRS) in Gram-positive bacteria (Wilcox, 1969a). In one of the latter, B. subtilis, both tRNA^{Glu} and tRNA^{Gln} are efficiently aminoacylated with glutamate (Wilcox and Nirenberg, 1968) by the same glutamyl-tRNA synthetase (GluRS) (Lapointe et al., 1986). The mischarged Glu-tRNA$^{Glu}$ is transformed into Gln-tRNA$^{Gln}$ by a specific amidotransferase (Wilcox, 1969b).

The GluRS and the GlnRS are also linked by the fact that, together with the arginyl-tRNA synthetase, they are the only aaRSs from E. coli which are unable to catalyze the formation of an aminoacyl-adenylate in the absence of their cognate tRNAs (Söll and Schimmel, 1974). Moreover, glutamine and arginine are produced biosynthetically from glutamate (Reitzer and Magasanik, 1987; Glansdorff, 1987). According to the theory of the co-evolution of the genetic code and of the amino acid biosynthetic pathways, glutamine and arginine occupy triplet codons allocated previously to glutamate, one of the few amino acids presumed to have been present in primordial proteins (Wong, 1975; for a review see Wong, 1988). Several groups of aaRSs specific for amino acids linked in their biosynthesis or/and occupying contiguous codons in the genetic code (the GluRS and the GlnRS (Breton et al., 1986), the leucyl-, isoleucyl-, methionyl-, and valyl-tRNA synthetases (Tzagoloff et al., 1988; Heck and Hatfield, 1986) and the aspartyl- and the lysyl-tRNA synthetases (Gampel and Tzagoloff, 1989)) present sequence similarities suggesting a common evolutionary history. In this context, the comparison of the primary structures of the GluRSs and the GlnRSs from E. coli with that of the GluRSs of Gram-positive bacteria may reveal structural elements involved in the catalysis of the aminoacylation reaction, in particular those involved in tRNA recognition.

In this report, we describe the cloning and the determination of the nucleotide sequences of the B. subtilis and Bacillus stearothermophilus gltX genes, the alignment of the primary structures of the GluRSs from B. subtilis, B. stearothermophilus, E. coli, and Rhizobium meliloti, and their comparison with those of the E. coli, Saccharomyces cerevisiae, and human GluRSs. We have identified a conserved amino acids sequence, shared only by the GluRSs of B. subtilis and of B. stearothermophilus and the GlnRSs of E. coli, which may be involved in the interaction between these enzymes and the anticodon region of their tRNA substrates. In the crystal structure of the E. coli glutamyl-tRNA synthetase complexed with tRNA$^{Glu}$ and ATP (Rould et al., 1989), residues
FIG. 3. Nucleotide and deduced amino acid sequences of B. subtilis and B. stearothermophilus gltX. The complete sequences of B. subtilis gltX (Bsu) are given, and differences between them and those of B. stearothermophilus (Bst) sequences are listed on two lines below. Series of three dots indicate gaps. The six internal fragments and NH₂- and COOH-terminal segments of the B. subtilis GluRS, whose amino acid sequences have been determined, are underlined.

EXPERIMENTAL PROCEDURES

RESULTS AND DISCUSSION

Partial Sequencing of B. subtilis Glutamyl-tRNA Synthetase—As no mixed-oligonucleotide designed from the NH₂-terminus of the glutamyl-tRNA synthetase of B. subtilis (Proulx et al., 1983) gave clear hybridization signal with the B. subtilis DNA, we sequenced internal peptides of the enzyme. Purified GluRS was digested with trypsin and six internal fragments were isolated and sequenced (see Fig. 3). Portions of this paper (including “Experimental Procedures,” part of “Results,” Table I, and Figs. 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

One of them, rich in residues corresponding to a small number of codons, was used to design one 50-mer unique ((Y₅) and two 17-mer mixed (α₅.1 and α₅.2) oligonucleotides (Table I). In the 50-mer oligonucleotide, the base assignment for the uncertain positions (degeneracy of the code) was done by using the statistical codon usage frequency of B. subtilis (Ogasawara, 1985; Shields and Sharp, 1987).

The NH₂-terminal amino acid sequence, as deduced from the nucleotide sequence, is Met-Gln-Asn-Glu-... (Fig. 3). Amino acid sequence analysis of the protein indicates the NH₂-terminal sequence to be Gly-Asn-Glu-... It appears that GluRS is synthesized with a NH₂-terminal methionine residue which is subsequently totally or partially removed. Digestion of the GluRS with carboxypeptidase A released isoleucine, asparagine, and small amounts of lysine and leucine. These are the last four amino acids in the COOH-terminal sequence (-Arg-Leu-Lys-Asn-Ile-COOH) deduced from the nucleotide sequence (shown in Fig. 3). As expected from the known from this conserved sequence near the anticodon of tRNA⁰₅⁰ and may interact with it.
Fig. 4. Amino acid alignments of glutamyl- and glutaminyl-tRNA synthetases. First, the glutamyl-tRNA synthetase sequences from R. me liloti (RmeGLuRS), E. coli (EcoGLuRS), B. subtilis (BsuGLuRS), and B. stearothermophilus (BstGLuRS) were aligned by the GAP program (cf. “Experimental Procedure”). Identical or conserved positions were boxed (gray boxes at positions where at least three GluRSs have an identical amino acid and white boxes for positions where at least three GluRSs have similar amino acids (Schwartz and Dayhoff, 1979)). Segments (I, II, and III) where we found, in over 60% of the positions, an identical amino acid in at least three GluRSs are indicated by bi-directional arrows. Short highly conserved motifs (motifs a–g) between the GluRSs are identified by (CIII). Then, each of the glutaminyl-tRNA synthetase sequences (E. coli (EcoGlnRS), human (HumGlnRS), and S. cerevisiae (SceGlnRS)) were individually compared with the GluRSs alignment. Gray boxes were extended to positions where an identity was found for one of the GlnRSs with at least three of the GluRSs. White boxes were extended to positions where a similar amino acid is found for one of the GlnRSs and at least three conserved amino acids of the GluRSs. The short highly conserved motifs between the GlnRSs are identified by (m). The levels of identity between these GluRSs are: Rme-Eco = 34%; Rme-Bsu = 38%; Rme-Bst = 38%; Eco-Bsu = 39%; Eco-Bst = 37%, and Bsu-Bst = 67%. Over the aligned part (G*-Tz9*), the E. coli GlnRS is about 20% identical to each of the four GluRSs.

specificity of carboxypeptidase A, arginine was not released.

Cloning and Sequencing of the gltX Gene of B. subtilis—Chromosomal DNA was digested with restriction endonucleases, separated by electrophoresis, and transferred to a nylon blotting membrane. Hybridization with the radiolabeled 50-mer oligonucleotide (Y 5 at 48 °C gave at least one strong signal and a few weaker signals for each digestion (Fig. 1A). With oligonucleotide Y 5.1 numerous weak signals appeared for every digestion (results not shown). Hybridization with probe Y 5.2 revealed for every digestion a unique and strong signal at approximately the same position as that of the stronger signal obtained with Y 5 (Fig. 1B).

We isolated DNA fragments of about 1.4 kbp generated by total digestion of chromosomal DNA with HindIII, including a fragment hybridizing with probe Y 5.2, and cloned them into vector pBS. We isolated four hybrid plasmids which had the same physical map (taking into account the orientation of the insert) and chose one of them, pLQB205, for further study. The sequencing strategy for pLQB205 is presented at the top of Fig. 2A. The nucleotide sequence of the entire insert is 1407 base pairs (158 base pairs of which are upstream from the ATG initiation codon) and encodes 418 amino acids of the GluRS, including its NH₂ terminus. The remainder of the gltX gene was identified by hybridization of the labeled insert of pLQB205 to a Southern transfer of genomic DNA digested with EcoRV (see Fig. 1C). Since the EcoRV site is near the beginning of the probe relative to the gltX gene, the stronger of the two hybridization signals (about 2.7 kbp, cf. Fig. 1C) was expected to contain the 3'-end of gltX. We also cloned the other hybridizing fragment to make sure that we had the regulatory region upstream gltX. An outline of the sequencing strategy for pLQB204 and pLQB206 is illustrated at the bottom of Fig. 2A. The entire inserts of these two plasmids were sequenced in order to determine the 3'-end sequence of the gltX gene together with the flanking sequences likely to play a role in its regulation (Fig. 3). The complete sequence of the gltX gene is presented in Fig. 3. The reading frame is 483 codons long and encodes six internal peptides isolated from the B. subtilis GluRS. The four amino acids released by partial digestion of the GluRS with carboxypeptidase A (Ile, Asn, Lys, and Leu) (see above) correspond to the four codons upstream of the end of gltX, indicating that translation of the

R. Breton and J. Lapointe, unpublished results.
using as a probe the insert of pLQB205 encoding the first 418 amino acids of the B. subtilis GluRS. This probe was used to hybridize on Southern blots of B. steurothermophilus DNA. The EcoRV digest (about 2.6 and 1.5 kbp) indicated that this signals per digestion (Fig. 1D). The presence of two signals in subtilis such as those for ribosomal proteins, is biased (Shields codon usage is not typical of the highly expressed genes. The motifs between the various GlnRSs (motifs a-g; see text and Fig. 4) are indicated by heavy lines. The conserved sequence between the E. coli GlnRS and the GluRSs of the two Bacillus species is indicated by an arrow.

GluRS messenger probably stops at the ochre codon shown in Fig. 3.

The gltX gene encodes a polypeptide whose molecular weight (55,671) has a significantly lower value than that (65,600) predicted by the mobility of the GluRS during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Proulx et al., 1983). Such a discrepancy has already been reported for many proteins (Burton et al., 1981; Price et al., 1983; Gitt et al., 1985; and Iacangelo et al., 1986) having a high negative charge density (Gitt et al., 1985), which is the case for the B. stearothermophilus aminoacyl-tRNA synthetases (this paper), E. coli (Breton et al., 1986), and R. meliloti (Laborge et al., 1989). Comparison of these sequences allowed the identification of several highly conserved segments that may be part of the active site or of substrate binding sites.

Fig. 4 shows the best primary sequence alignment of these four GluRSs obtained using the computer program GAP (cf. "Experimental Procedures"). By introducing only a few gaps at scattered places, the four sequences have an identity of 20%, whereas 44% of the positions are identical for at least three GluRSs (gray boxes in Fig. 4). If we take into account the amino acids having similarity in their functional groups (Schwartz and Dayhoff, 1979), 39% of the overall positions are conserved by the four GluRSs and 67% by at least 3 of them (white and gray boxes in Fig. 4). This alignment reveals three long segments (I, II, and III) where, in over 60% of the positions, an identical amino acid was present in at least three out of four proteins. These segments are interrupted by two intersegments with no significant similarity (less than 25% identity of 30% whereas 62% of the residues are identical in 471), and the four GluRS sequences have, in these regions, an identity of 30% whereas 62% of the residues are identical in at least 3 of them. This suggests that these parts of the GluRSs are likely to play a role in catalytic activity.

Primary Structure Comparison between the Four GluRSs and the E. coli, Saccharomyces cerevisiae, and Human Glutaminyl-tRNA Synthetases—To the alignment of the GluRSs, we have added the primary structures of the E. coli (Yamao et al., 1982), S. cerevisiae (Ludmerer and Schimmel, 1987) and human glutaminyl-tRNA synthetases (Thoennes et al., 1988) in order to find conserved elements indicative of a close
**FIG. 6.** Comparison of the relative locations of several short highly conserved sequences identified in the glutamyl-tRNA synthetases from different organisms with those found in other aminoacyl-tRNA synthetases. These sequences correspond to or are part of the highly conserved motifs (motifs a, b, d, e, f, and g) observed by comparing the primary structures of several GluRSs (cf. Fig. 4). The numbers in brackets indicate the number of amino acids separating motif a from the NH₂ end of the synthetase or separating two motifs. Dashed boxes indicate homology between motifs for aminoacyl-tRNA synthetases specific for different amino acids. Abbreviated names for aaRSs are: IleRS, isoleucyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; MetRS, methionyl-tRNA synthetase; ValRS, valyl-tRNA synthetase; TyrRS, tyrosyl-tRNA synthetase; TrpRS, tryptophanyl-tRNA synthetase. Abbreviated names for organisms are: Eco, E. coli; Rme, R. meliloti; Bsu, B. subtilis; Bst, B. stearothermophilus; Bca, B. caldotenax; See, S. cerevisiae; Hum, human. GluRS(Eco), Webster et al. (1984); GluRS(Sce), Englisch et al. (1987); LeuRS(Eco), Haertlein and Madern (1987); LeuRS(Sce), Tzagoloff et al. (1988); MetRS(Eco), Barker et al. (1982a); MetRS(Sce), Walter et al. (1983); ValRS(Bst), Borgford et al. (1987); ValRS(Eco), Haertlein et al. (1987); ValRS(Sce), Jordana et al. (1987); TyrRS(Bst), Jones et al. (1986); TyrRS(Bst), Winter et al. (1983); TrpRS(Eco), Barker et al. (1982b); TrpRS(Bst), Barstow et al. (1986); TrpRS(Eco), Hall et al. (1982).
evolutionary linkage between these two enzymes. Such an alignment between the GluRS and GlnRS of E. coli has already been reported (Breton et al., 1986). Nevertheless, the availability of primary structures for both enzymes from many evolutionary distant organisms permits a better alignment over a longer sequence.

Fig. 4 shows this alignment which goes from the beginning of the GluRSs sequences and covers more than the NH2-terminal half of these proteins (269/471 amino acids for the E. coli GluRS) with only a few insertions or deletions. Considering only positions where at least two GlnRSs have an identical or a similar (with values ≥ 0.5 in the Dayhoff table (Schwartz and Dayhoff, 1979) recaled by Gribskov and Burgess (1986)) amino acid with at least two GluRSs, 44% of all positions in this region are conserved. These conserved positions are especially grouped in a region corresponding to the first 110 amino acids of the E. coli GluRS where the level of conserved residues reaches 63%. Finally in the GlnRSs sequences, the motif VKKSKR matches that found in the GluRS (KLASKR). When we align these two motifs, a sequence, conserved among the GlnRSs (GWDDPRK), matches with that of GluRSs (GYLPEAK). From this position on, no significant homology was detected in the COOH-terminal half between the four GluRSs and three GlnRSs, even when allowing many gaps. The alignment covers entirely the two halves of the dinucleotide binding domain and the acceptor binding domain and about half of the helical subdomain in the three-dimensional structure of the E. coli GlnRS (Rould et al., 1989). The strong homology in this region suggests that the tertiary structure of the NH2-half of the GluRS is similar to that reported for the GlnRS.

Considering that B. subtilis GluRS aminoacylates efficiently its own tRNA\textsubscript{Glu} in \textit{vitro} and one of the two tRNA\textsubscript{Gln} isoacceptors of \textit{E. coli} in \textit{vitro} (Lapointe et al., 1986), any conserved regions between the GluRSs of the two Bacillus species and the E. coli GlnRS which are not conserved with the GluRSs of \textit{E. coli} and \textit{R. meliloti} could be involved in the recognition and binding of tRNA\textsubscript{Glu}. The only region endowed with this property is a sequence of 30 amino acids in B. subtilis (N\textsuperscript{Glu} \textsuperscript{120}E\textsuperscript{124}) and \textit{D. stearothermophilus} (N\textsuperscript{Glu} \textsuperscript{307}D\textsuperscript{311}) GluRSs, similar to the N\textsuperscript{Glu} \textsuperscript{20}D\textsuperscript{24} region in the COOH-terminal half of the E. coli GlnRS (Fig. 4). Indeed, 45% of the GlnRS residues are identical with those of at least one of these two GluRSs; including the similar amino acids (see above), 72% of this sequence is conserved. Interestingly, in the three-dimensional structure of the GlnRS, this sequence belongs to the distal \beta-barrel domain, a region found to be at close proximity to the anticodon nucleotides of tRNA\textsubscript{Glu} (Rould et al., 1989; see heavy line indicated by an arrow in Fig. 5). Since these nucleotides play a role in the recognition of tRNA\textsubscript{Glu} by the GlnRS, this homology between the GlnRSs of \textit{E. coli} and the GluRSs of these \textit{Glu}\textsuperscript{+} positive bacteria indicates that conserved residues in this region of 30 amino acids are probably involved in specific interactions between these enzymes and the anticodon regions of their tRNA substrates. Residues located in loops at the beginning and near the middle of this conserved sequence are in the vicinity of the anticodon of tRNA\textsubscript{Glu}, which are not recognized \textit{in vitro} by the \textit{B. subtilis} GluRS, \textit{i.e.} \textit{B. subtilis} tRNA\textsubscript{Glu}\textsuperscript{Glu} and tRNA\textsubscript{Glu}, and since at position 34 the three tRNAs recognized \textit{in vitro} by the \textit{B. subtilis} GluRS, \textit{i.e.} \textit{B. subtilis} tRNA\textsubscript{Glu}\textsuperscript{Glu} and tRNA\textsubscript{Glu}, \textit{E. coli} tRNA\textsubscript{Glu}\textsubscript{Glu} have a U34, whereas \textit{E. coli} tRNA\textsubscript{Glu}\textsubscript{Glu} and \textit{E. coli} tRNA\textsubscript{Glu}\textsubscript{Glu} which are not recognized \textit{in vitro} by this GluRS, have, respectively, a C34 and a modified U34 (Lapointe et al., 1986).

Identification of Conserved Sequences among a Group of Aminoacyl-tRNA Synthetases—Alignment of the four GluRSs reveals the presence of seven short and highly conserved stretches of amino acids (motifs a–g) (Fig. 6), all localized in the first two segments (see Fig. 4). These motifs could represent functionally conserved regions. Two of them (motifs a and f) correspond to regions of homology already reported for other aminoacyl-tRNA synthetases: the signature sequence (Webster et al., 1984) and the consensus KMSKS sequence (Hountondji et al., 1986), respectively. Such highly conserved motifs are also present in other aaRSs whose primary structure is known for several organisms. Comparison of the relative locations and amino acid sequences of these motifs could be indicative of the functions of these motifs and of evolutionary relations among these enzymes.

The binding site for tyrosine on the tyrosyl-tRNA synthetase (Brick and Blow, 1987; Brick et al., 1988) is formed by residues from several regions of the enzyme. These residues mostly belong to four short sequences highly conserved among TyrRSs from various species. When the homologous signature and KMSKS sequences of the GluRSs are aligned with those of the TyrRSs, the four short sequences occupy sites corresponding to motifs a, b, d, and e of the GluRSs (Fig. 6). The first two sequences (corresponding to motifs a and b) are near the NH2-terminal end of all TyrRSs and are separated by 17 amino acids, whereas the other two (motifs d and e), separated by 10 amino acids, are 20 amino acids upstream of a conserved sequence similar to KMSKS. A similar arrangement is found for several other synthetases (Fig. 6). If these synthetases have a three-dimensional structure analogous to that of the TyrRS, these motifs could occupy the same positions and could have the same function, as demonstrated for one residue located in motif b) of the \textit{E. coli} isoleucyl-tRNA synthetase (Fig. 6) and shown to be involved in isoleucine binding (Burbank et al., 1989). By analogy with structural studies of the tyrosyl- and isoleucyl-tRNA synthetases, it is possible that the conserved motifs a, b, d, and e could form the binding site for glutamate on GluRS.

The amino acid sequences of these motifs could be indicative of an evolutionary linkage between aaRSs specific for distinct amino acid substrates. Indeed, subsets of at least 8 aaRSs, which share the signature and the KMSKS sequences, also share other motifs (Fig. 6). Motif h from isoleucyl-, leucyl-, methionyl-, and valyl-tRNA synthetases is very conserved, suggesting that these enzymes form a subclass of synthetases that evolved from the same ancestor (Tzagoloff et al., 1988, Heck and Hatfield, 1988). The homology between glutamyl- and glutaminyl-tRNA synthetases (motifs b, d, and e), is still stronger and is consistent with a close evolutionary linkage between these two enzymes. In the crystal structure of the glutaminyl-tRNA synthetase complexed with tRNA\textsubscript{Glu} and ATP (Rould et al., 1989), motifs a, b, d, and f are near the ATP or the 3' end of tRNA\textsubscript{Glu} (Fig. 5). Motifs c and g are at some distance from these substrates, but their conservation suggests that they could play a role in the catalytic activity of the glutaminyl-tRNA synthetase and probably also in that of the GluRS.
REFERENCES


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GLUTAMYL-tRNA SYNTHETASES OF TWO BACILLUS SPECIES

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SUGGESTED MATERIAL

Materials: All restriction endonucleases and modification enzymes were purchased from New England BioLabs, Inc. Plasmids were isolated using the alkaline lysis procedure of Birnboim and Doly (1979). Cultures were grown in the presence of chloramphenicol as indicated in the text, and in the last step of the synthesis the plasmid was linearized with the enzyme SacI. DNA from Bacillus subtilis was prepared in the laboratory of Robert M. Ochman. The DNA from B. stearothermophilus was isolated as described by Ochman et al. (1981). The DNA from B. subtilis and B. stearothermophilus was isolated as described by Ochman et al. (1981).


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gltX Genes for Glutamyl-tRNA Synthetases of Two Bacillus Species

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Glutamyl-tRNA synthetases of Bacillus subtilis 168T and of Bacillus stearothermophilus. Cloning and sequencing of the gltX genes and comparison with other aminoacyl-tRNA synthetases.
R Breton, D Watson, M Yaguchi and J Lapointe