Molecular Analysis of a Novel Glutamine Synthetase of the Anaerobe *Bacteroides fragilis*

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(Received 19 June 1989; revised 31 August 1989; accepted 18 September 1989)

The nucleotide sequence of a 2777 bp DNA segment containing the *Bacteroides fragilis glnA* gene was determined. The *B. fragilis glnA* open reading frame of 2187 bp encoded a glutamine synthetase (GS) subunit of 729 amino acid residues with a calculated M_r of 82827. The apparent M_r of the GS subunit determined by SDS-PAGE was approximately 75000. A single mRNA transcription start point was identified upstream of the *B. fragilis glnA* open reading frame. The *B. fragilis* GS subunit is approximately 270 and 400 amino acids longer than the GSI and GSII subunits, respectively, of other prokaryotes and eukaryotes. The GSI and GSII holoenzymes are dodecamers and octamers respectively, whereas the GS of *B. fragilis* is a hexamer. Although GSI and GSII subunits show amino acid similarity in five conserved regions, this similarity is not strongly conserved in the *B. fragilis* GS. The GS of *B. fragilis* is not regulated by adenylation and lacks the adenylation site. It also lacks the Trp residue associated with the active site in GSI and GSII enzymes from other prokaryotes and eukaryotes.

INTRODUCTION

Glutamine synthetase (GS) (EC 6.3.1.2) is an important enzyme in nitrogen metabolism and ammonia assimilation in many prokaryotes, eukaryotes and archaeobacteria. GS enzymes of eukaryotic origin are octamers (Prusiner & Stadtman, 1973) whereas the GS enzymes of eubacteria and an archaeobacterium are dodecamers of M_r approximately 600000 composed of a single type of subunit, the M_r of which falls in the range 44000 to 59000 (Streicher & Tyler, 1980; Bhatnagar *et al.*, 1986).

Prokaryotes have two forms of GS, termed GSI and GSII. The majority of bacteria investigated have GSI enzymes but members of the *Rhizobiaceae* contain both GSI and GSII enzymes. GSI is the typical prokaryotic GS whereas GSII is similar to eukaryotic GS enzymes (Carlson & Chelm, 1986). Prokaryotic GSI subunits investigated vary in length from 444 to 474 amino acids (Janssen *et al.*, 1988), whereas the GSII subunits of eukaryotes investigated vary in length from 355 to 373 amino acids (Gebhardt *et al.*, 1986; Hayward *et al.*, 1986; Tischer *et al.*, 1986). The eukaryotic GSII subunits lack the C-terminal portion of the prokaryotic GSI subunit including the adenylation site. The GSII subunit from *Bradyrhizobium japonicum* is 329 amino acids long and also lacks the terminal portion of the GSI subunit and the adenylation site (Carlson & Chelm, 1986). In a comparison of prokaryotic and eukaryotic GSI and GSII enzymes, Rawlings *et al.* (1987) showed that although the amino acid similarity between the enzymes was only approximately 15%, the major part of this similarity was located in five

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Abbreviations: *glnA*, gene encoding glutamine synthetase; GS, glutamine synthetase; ORF, open reading frame; SD, Shine–Dalgarno.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M28252.

regions corresponding to β -sheets in the *Salmonella typhimurium* enzyme (Almassy *et al.*, 1986). These regions are strongly conserved in all GS enzymes analysed to date (Janssen *et al.*, 1988). A feature of the five conserved amino acid regions is that they are all associated with the proposed GS active site (Almassy *et al.*, 1986; Janson *et al.*, 1986). Regions II to V are β -strands closely associated with two Mn^{2+} cations of one subunit, while region I contains the Trp residue which is thought to complete the active site formed between adjacent subunits.

An indication that the GS from the Gram-negative obligately anaerobic bacterium *Bacteroides fragilis* may differ from other GS enzymes of eukaryotes and prokaryotes was suggested by Southern *et al.* (1986, 1987). Studies of the cloned *B. fragilis* GS subunit produced in *Escherichia coli* and *B. fragilis* indicated that the *B. fragilis* GS subunit was substantially larger (M_r approximately 75000) than other GSI and GSII subunits (maximum M_r approximately 59000). However, the M_r of the *B. fragilis* GS holoenzyme (approximately 490000) was lower than that of the prokaryotic GSII holoenzyme (approximately 600000). It was concluded that the GS of this anaerobic prokaryote is a hexamer and not a dodecamer. To confirm the structure of the *B. fragilis* GS we sequenced the *glnA* gene and compared the nucleotide sequence and the derived amino acid sequence with other GSI and GSII enzymes. We report that the GS from *B. fragilis* differs markedly from the GSI and GSII enzymes from other prokaryotes and eukaryotes.

METHODS

Bacterial strains, plasmids and growth conditions. *B. fragilis* strain Bf-1 was described by Mossie *et al.* (1979) and was used by Southern *et al.* (1986, 1987) for the cloning of the *B. fragilis glnA* gene. Plasmid pJS139 [GlnA⁺, ampicillin resistant (Ap^r)], is an *E. coli* recombinant plasmid which contains the *B. fragilis* Bf-1 *glnA* gene and enables *E. coli glnA* deletion mutants to utilize $(NH_4)_2SO_4$ as a sole source of nitrogen (Southern *et al.*, 1986, 1987). *E. coli* strains were grown on Luria medium (Miller, 1972) or glucose minimal medium (Miller, 1972) with 1 g $(NH_4)_2SO_4$ per litre as the sole nitrogen source. All strains were grown at 37 °C with aeration.

Preparation of DNA. Plasmid DNA was prepared by the alkali-hydrolysis method of Ish-Horowitz & Burke (1981).

DNA sequence analysis. DNA sequencing of both strands of plasmids containing the cloned *B. fragilis glnA* gene was done by the dideoxynucleotide triphosphate chain termination method of Sanger *et al.* (1977) with templates prepared from DNA subcloned in pUC18 and pUC19 and exonuclease III digestion (Henikoff, 1984). *E. coli* LK111 (Zabeau & Stanley, 1982) was used as the recipient strain. The DNA chains were radiolabelled with [³⁵S]dCTP (400 Ci mmol⁻¹; 14.8 TBq mmol⁻¹). The DNA and deduced amino acid sequences were analysed with an IBM XT computer using a GENPRO (version 3.1) program (Riverside Scientific).

Primer extension reactions. A synthetic 17-mer oligonucleotide 5'ATAGCTCTGTAGTGCG-3' was used as a primer for the synthesis of cDNA. A [α -³²P]-5'-end-labelled primer (5 ng) was mixed with 10 μ g of RNA prepared from *B. fragilis* Bf-1 or *E. coli* LK111(pJS139) by the method of Aiba *et al.* (1981). The mixture was denatured at 95 °C in 10 mM-Tris/HCl pH 8.3, 0.35 mM-EDTA for 5 min and chilled on dry ice. Primer-RNA hybridizations were incubated at 42 °C for 6 h. The reverse transcription reaction was carried out at 42 °C for 60 min in 20 μ l of 50 mM-Tris/HCl, pH 8.3, 8 mM-MgCl₂, 2 mM-DTT, 1 mM each of dGTP, dATP, dCTP, dTTP and 50 units of reverse transcriptase. The RNA was degraded with RNAase A (20 μ g) for 10 min at 37 °C. After phenol extraction and ethanol precipitation the cDNA was resuspended in 10 μ l of 99% (w/v) formamide, 0.1 mg bromophenol blue ml⁻¹, 0.1 mg xylene cyanol ml⁻¹ and electrophoresed in a sequencing gel.

RESULTS AND DISCUSSION

Nucleotide sequence and transcription start point of *glnA* gene

The nucleotide sequence of a 2777 bp region of *B. fragilis* DNA containing the *glnA* gene was determined. The DNA sequence contained an open reading frame (ORF), which from a start codon (ATG) to a stop codon contained 2187 nucleotides (Fig. 1). Primer extension experiments carried out with RNA isolated from *B. fragilis* cells grown in brain heart infusion broth

Fig. 1 (on facing page). Nucleotide sequence for the *glnA* gene of *B. fragilis* with up- and downstream regions. The derived amino acid sequence is given in the one-letter code from positions 1 to 2187 (729 residues). The -10 and -35 regions of the putative promoter are in boldface type and underlined. The putative Shine-Dalgarno (SD) sequence AAAAGAGA starts at position -8. Two near-perfect direct repeat sequences from positions -127 to -82 and -74 to -30 are marked with lines above the sequences.

-300 -250
 CAGAGATGGGACGATGGTCTGACCATCAAGAAACACGTTAGCCACGCCCTTTTGAACAAACAGAGCGAGATAACCAAAAAACCATATTAATATATTGCACCTCA
 -200 -150
 AATACTAATCTCCTTATTGAGGCAATATAAAAAATCCGATTATGCGACTAATAAATTCATATTTTATAGTATCTCCGATAGTAAAGTATCAAAAAAGTTCTTTGCCCAAAA
 -100 -50
 KCAAAATAGGCGCTACAAAGAAAAACATATTTATTTTGCAAAATACCCCTATAAAAAATAGGGGTATATTTGAACAAAAACATATAATTTTGTGCCCAACACCAATATTAAGAGACCAACATC
 -35 -10 50
 1 ATG TCA AAA ATG AGA TTT TTC GCA CTA CAA GAG CTA TCT AAC AGA AAG CCC TTA GAA ATT ACT ACT CCC TCT AAT AAA CTA TCC GAT TAT
 M S K M R F F A L Q E L S N R K P L E I T T P S N K L S D Y
 91 TAT GCC AGC CAT GTA TTC GAT CGT AAG AAG ATG CAA GAG TAC CTT CCC AAA GAA GCA TAC AAA GCA GTA GTA GAC GCT ACC GAA AAA GGC
 Y A S H V F D R K K M Q E Y L P K E A Y K A V V D A T E K G
 181 ACC CTT ATC AGC CGA GAA ATG GCC GAC CTG ATA GCC AAC GGC ATG AAA AGC TGG GCA AAA TCG CTC AAT GTG ACA CAC TAC ACG CAC TGG
 T P I S R M A L I A N G M K S W A K S L N V D A T H Y T H W
 271 TTT CAG CCA CTG ACA GAC GGA ACG GCC GAG AAA CAT GAC GGC TTT ATC GAG TTC GGT GAA GAC GGT GAA GTA ATC GAA CGT TTT TCC GGC
 F Q P L T D G T A E K H D G F I E F G E D G E V I E R F S G
 361 AAA TTA CTG ATC CAG CAG GAA CCG GAC GCT TCT TCT TTC CTT AAT GGT GGA ATC CGT AAT ACA TTC GAA CGC CGC GGT TAC ACT GCA TGG
 K L L I Q Q E P D A S S F P N G G I R N T F E A R G Y T A W
 451 GAC GGT TGG TCA CCG GCT TTC GTA GTA GAC ACC ACT CTT TGT ATC CCG ACG ATT TTT ATC TCA TAT ACA GGT GAA GCA CTC GAC TAC AAA
 D G S S P A F V V D T T L C I P T I F I S Y T G E A L D Y K
 541 ACT CCA TTA TTA AAA GCA TTG GCA GCC GTT GAC AAA GCT GCT ACC GAA GTA TGC CAG CTT TTC GAC AAA AAC ATC ACC CGG GTA TTC ACC
 T P L L K A L A A V D K A A T E V C Q L F D K N I T R V F T
 631 AAT TTG GGT TGG GAA CAG GAA TAT TTT TTG GTT GAC ACC TCT TTA TAT AAT GCC CGC CCC GAC CTT CGA CTA ACA GGA CGC ACC TTG ATG
 N L G W E Q E Y F L V D T S L Y N A R P D L T G R L T M H
 721 GGA CAT TCT TCG GCC AAG GAC CAA CAG TTG GAA GAT CAT TAC TTC GGT TCT ATT CCA CCA GGC CTC ACA GCT TTC ATG AAA GAA CTT GAA
 G H S S A K D Q Q L E D H Y F G S I P P R V T A F M K E L E
 811 ATA GAG TGT CAC AAG CTG GGC ATA CCG GTA AAG ACC CGC CAC AAT GAA GTC GCT CCC AAC CAG TTT GAA TTG GCT CCC ATT TTT GAA AAC
 I E C H K L G I P V K T R H N E V A P N Q F E L A P I F E N
 901 TGT AAT CTG GCG AAT GAC CAC AAT CAG TTG GTA ATG GAC CTG ATG AAA CGT ATC GCA CGC AAA CAT CAT TTT GCT GTT CTT TTC CAT GAA
 C N L A N D H N Q L V M D L M K R I A R K H H F A V L F H E
 991 AAG CCC TAC AAT GCC GTT AAT GGT TCG GGC AAA CAT AAC AAC TGG TCG CTC TGC ACC GAT ACA GGC ATC AAC CTC TTT GCA CGC GGT AAA
 K P Y N G V N G S G K H N N W S L C T D T G I N L F A P G K
 1 081 AAT CCA AAA GGA AAC ATG CTA TTC CTT ACT TTC CTG GTA AAC GTA TTA ATG ATG GTC CAC AAG AAT CAG GAT CTG TTG CGT GCT TCT ATT
 N P K G W E Q E Y F L T F L V N V L M M V H K N Q D L L R A S I
 1 171 ATG AGT GCC GGA AAC AGT CAC CGT TTG GGA GCC AAC GAA CCA CCT CCC GCT ATC TTG TCC ATT TTC CTG TGC CAA CTG TCA GCT ACA
 M S A G M S H R L G A N E A P P A I L S I F L G S Q L S A T
 1 261 CTC GAT GAA ATT GTC CGT CAG GTG ACC AAC TCA AAA ATG ACT CCA GAG GAG AAG ACA ACT TTG AAA TTA GGT ATC GGG CGT ATA CCC GAA
 L D E I V R Q V T N S K M T P E E K T T L K L G I G R I P E
 1 351 ATC CTG CTC GAT ACC ACC GAC CGT AAT CGC ACT TCT CTT TCA TTC ACA GGA AAT CGT TTT GAG TTC CGT GCC GCC GGC TCC TCA GCC
 I L L D T T D R N R T S P F A F T G N R F E F R A A G S S A
 1 441 AAC TGT GCT GCT GCC ATG ATT GCC ATA AAT GCT GCC ATG GCC AAC CAG TTG AAT GAA TTT AAA GCA TCG GTA GAC AAA CTG ATG GAA GAG
 N C A A A M I A I N A A M A N Q L N E F K A S V D K L H E E
 1 531 GGT ATC GGT AAG GAC GAA GCA ATC TTC CGC ATC CTG AAA GAA AAT ATC ATA GCC TCC GAA CTT ATC CGT TTT GAG GGT GAC GGC TAC TCA
 G I G K D E A I F R J L K E N I I A S E L I R F E G D G Y S
 1 621 GAA GAG TGG AAA CAG GAA GCA GCC CGC GGA CTA ACC AAC ATC TGC CAT GTT CCG GAG GCC CTG ATG CAT TAT ATG GAT AAC CAA TCG
 E E W K Q E A A R R G L T M I C H V P E A L M H Y A L D N Q S
 1 711 AGA GCC GTA CTG ATA GGC CAA GGT ATC TTC AAC GAA ACC GAA CTC GCC TGT CGT CTG GAA CTG GAA TTG GAA AAA TAT ACC ATG AAG GTA
 R A V L I G E R I F N E T E L A C R L E V E L E K Y T M K V
 1 801 CAG ATT GAA AGC CGC GTA TTG GGT GAT CTG GCC ATC AAC CAC ATC GTC CCT ATC GCT GTC AGC TAC CAG AAC CGT CTT TTG GAA AAC CTT
 Q I E S R V L G D L A I N H I V P I A V S Y Q N R L L E N L
 1 891 TGC AGA ATG AAG GAA ATC TCT TCT GAA GAA TAC GAA GTA ATG AGT GCC GAT CGG AAA GAA CTT ATT AAA GAA ATC TCT CAC CGT GTA
 C H M K E I F S E E E Y E V M S A D R K E L I K E I S H R V
 1 981 TCT GCT ATT AAA GTA CTG GTA CGC GAC ATG ACA GAA GCC CGC AAA GTA GCC AAT CAC AAA GAG AAC TTC AAA GAG AAA GCT TTT GCT TAC
 S A I K V L V R D M T E A R K V A N H K E N F K E K A F A Y
 2 071 GAA GAG ACC GTA CGT CCT TAC CTG GAA AGC ATA CGC GAC CAT ATA GAC CAT CTC GAG ATG GAA ATT GAC GAT GAA ATT TGG CCG TTG CCC
 E E W R P Y L E S I R D H I D H L E M E I D G E I W P L P
 2 161 AAA TAC AGA GAA CTG TTA TTC ACG AAG TAA GCCATACTGATGTCTCCCGTAAGAAGTCTCTTTCAAGATCTCTTATGGGAGCTTTTCATTTCCGGGGTTTCCTC
 K Y R E L L F T K *

TACCCAACCCCTACTTTCTCGCCGCCAACATCCACGGTCTCTCAACAAAAGATTGAAAGAGATCGGAGTACACGCATGCACAAAGTTAAAGCAGATTTCAGGTTCACATTATANCAT

ATCCCACTGCTGTTCCGTCATACGCATCATCATCCGTCACG

Fig. 2. Transcript mapping of *glnA* in mRNA from *B. fragilis* (lane B). Lanes A, C, G and T represent the dideoxy sequencing reaction products of pJS139 DNA primed with the synthetic primer. The 5' end of the *glnA* transcript was determined by primer extension. The transcription start point is indicated with an arrow alongside the corresponding DNA sequence.

(Holdeman & Moore, 1972) showed a single major transcription start point 18 bp from the first nucleotide of the ATG start codon (position + 1) of the 2187 bp ORF (Fig. 2). Similar primer extension experiments carried out with RNA isolated from *E. coli* (pJS139) cells showed a number of weak transcription start points within 16 to 40 bp of the ATG start codon (results not shown). Although promoter consensus sequences for *B. fragilis* have not been defined, it is interesting that an *E. coli* promoter sequence containing -10 and -35 RNA-polymerase-binding consensus sequences separated by 22 bp is situated 32 bp upstream of the *B. fragilis* transcription start point (Fig. 1). Previously we reported that the apparent M_r of the *B. fragilis* GS subunit determined by SDS-PAGE was approximately 75000 (Southern *et al.*, 1986, 1987). The start region of the *B. fragilis glnA* ORF contains five in-phase ATG start codons which would result in proteins with calculated M_r values of 82827, 82481, 78067, 75131 and 74345 respectively (Fig. 1). The next downstream in-phase ATG codon would produce a protein with a calculated M_r of 55823, which is too small to be regarded as the *B. fragilis* GS. The first ATG codon is preceded by an AAGAGA sequence eight nucleotides from the presumptive start codon. This AG-rich region shows some similarity to other Shine-Dalgarno (SD) sequences (Shine & Dalgarno, 1976) and is situated close enough to the start of the ORF to be regarded as a putative SD region. The other four ATG codons were not preceded by sequences which showed similarity to SD sequences. The *B. fragilis glnA* gene was expressed in *E. coli* from a promoter site contained within the cloned *B. fragilis* DNA (Southern *et al.*, 1986). Attempts to sequence the amino-terminal end of the GS subunit were unsuccessful as the protein appeared to be blocked. A feature of the upstream region is the presence of two near-perfect direct repeat sequences of 46 and 45 bp from positions -127 to -82 and -74 to -30 respectively (Fig. 1). This region of direct repeats overlaps the putative promoter sequence and may be involved in the regulation of GS expression. The regulation of the *glnA* gene in *Salmonella typhimurium* and *E. coli* involves five NR₁ binding consensus sequences of 15 bp (Ames & Nikaido, 1985). Some of the NR₁ binding sites overlap the *glnA* promoters. However, there is no homology between the repeats upstream of the *B. fragilis glnA* gene and the NR₁ binding consensus sequences.

Comparison of B. fragilis GS with other GS enzymes

Recently Rawlings *et al.* (1987), Janssen *et al.* (1988), Strauch *et al.* (1988) and Wray & Fisher (1988) have compared the nucleotide-sequence-derived GS amino acid sequences from diverse prokaryotes including *Bradyrhizobium japonicum* (Carlson & Chelm, 1986), *E. coli* (Colombo & Villafranca, 1986), *Anabaena* sp. strain 7120 (Tumer *et al.*, 1983), *Thiobacillus ferrooxidans* (Rawlings *et al.*, 1987), *Salmonella typhimurium* (Janson *et al.*, 1986), *Clostridium acetobutylicum*

Fig. 3. Dot-matrix comparisons of amino acid sequences of GSI enzymes from *Salmonella typhimurium* and *Streptomyces coelicolor*, of GSII enzymes from alfalfa and *Bradyrhizobium japonicum*, and of the *Bacteroides fragilis* GS with a GSI enzyme and a GSII enzyme. Dot-matrix comparisons were done using the GENEPRO (version 3.1) sequence analysis program (Riverside Scientific), checking for five matches in a window of ten residues.

(Janssen *et al.*, 1988), *Bacillus subtilis* (Strauch *et al.*, 1988), *Azospirillum brasilense* (Bozouklian & Elmerich, 1986) and *Streptomyces coelicolor* (Wray & Fisher, 1988).

The GSII subunit from *Bradyrhizobium japonicum* contained 329 amino acids (M_r 36865), whereas the GSI subunits of the other bacteria contained 444 to 474 amino acids (M_r 49000 to 59000). In contrast, the *Bacteroides fragilis* *glnA* ORF of 2187 nucleotides encodes a GS subunit of 729 amino acid residues with a calculated M_r of 82827. Thus the *B. fragilis* GS subunit is approximately 270 and 400 amino acids longer than the GSI and GSII subunits respectively. The calculated M_r of the *B. fragilis* GS confirms our previous results (Southern *et al.*, 1987) involving PAGE analysis of the GS which indicated that the *B. fragilis* GS subunit was substantially larger than other prokaryote GS subunits.

The GSI and GSII enzymes can be separated into two groups on the basis of amino acid similarity. Although there is only limited amino acid similarity between these two groups, a high degree of similarity exists between GS enzymes within each group. This is illustrated by the dot-matrix comparisons of GS subunits from distantly related organisms within each group (Fig. 3). The GS from *B. fragilis* did not show any overall amino acid similarity with either GSI or GSII enzymes (Fig. 3).

The major regions of amino acid sequence similarity between the prokaryote GS enzymes involve five β -sheets which are all associated with the proposed GS active site (Almassy *et al.*, 1986; Janson *et al.*, 1986; Rawlings *et al.*, 1987). These regions are also conserved in GS from eukaryotes (Janssen *et al.*, 1988). A comparison of these five regions from representative groups of eukaryotes, prokaryotes and *B. fragilis* is shown in Fig. 4. In the GS enzyme from *B. fragilis*

REGION I

Hs	60	N	F	D	G	S	S	T	L	Q	S	E	G	S	N	-	S	D	M	Y	L	V	P	A	A	-	-	M	F	R	D	P	F	R
Af	54	N	Y	D	G	S	S	T	G	Q	A	P	G	E	D	-	S	E	V	I	I	Y	P	Q	A	-	-	I	F	F	K	D	A	R
Bj	42	G	F	D	G	S	S	T	Q	Q	A	E	G	H	S	-	S	D	C	V	L	K	P	V	A	-	-	V	F	F	D	A	A	R
An	50	P	F	D	G	S	S	I	R	G	W	K	A	I	N	E	S	D	M	T	M	V	L	D	P	N	T	A	W	I	D	P	F	F
St	49	M	F	D	G	S	S	I	G	G	W	K	G	I	N	E	S	D	M	V	L	M	P	D	A	S	T	A	V	I	D	P	F	F
Tf	50	S	F	D	G	S	S	I	A	G	W	K	G	I	N	E	S	D	M	I	L	L	P	D	P	D	S	A	V	I	D	P	F	F
Ca	51	M	F	D	G	S	S	I	D	G	F	V	R	I	E	E	S	D	M	N	L	R	P	N	L	S	T	F	V	I	F	P	F	R
Sc	48	A	F	D	G	S	S	I	R	G	F	Q	A	I	H	E	S	D	M	S	L	R	P	D	L	S	T	A	R	V	I	F	P	F
Bs	51	M	F	D	G	S	S	I	E	G	F	V	R	I	F	E	S	D	M	V	L	V	P	D	L	N	T	F	V	I	F	P	F	R
Bf	149	A	W	D	G	S	S	P	A	F	V	V	D	T	T	L	C	I	P	T	I	F	I	S	Y	T	G	E	A	L	D	Y	K	T

REGION II

Hs	195			V	V	-	-	Q	Q	E	E	F	Q	I
Af	191			E	E	-	-	A	W	W	E	F	Q	V
Bj	171			V	V	P	P	K	C	C	E	L	G	F
An	215			V	V	T	T	T	N	N	E	V	A	T
St	215			V	V	A	A	G	A	A	E	E	E	E
Tf	211			V	V	G	G	G	G	G	E	E	E	E
Ca	187			V	V	A	A	-	-	-	E	E	E	E
Sc	212			V	V	A	A	G	A	G	E	E	E	E
Bs	188			V	V	T	T	T	T	T	E	E	E	E
Bf	285			V	V	P	P	-	-	-	E	E	E	E
		A	G	A	G	A	G	A	G	A	A	H	F	P
		E	E	E	E	E	E	E	E	E	E	E	E	E
		H	H	H	H	H	H	H	H	H	H	H	H	H
		N	E	E	E	E	E	E	E	E	E	E	E	E

REGION III

Hs	237	T	S	L	D	P	K	P	I	P	G	N	-	W	N	G	A	G	C	H	T	N	F	S	T		
Af	233	S	F	D	P	K	K	K	K	K	G	D	-	W	N	G	A	A	G	A	H	T	N	Y	S	T	
Bj	213	E	F	H	C	K	P	L	G	D	T	D	-	W	N	G	S	G	M	H	A	N	F	S	T		
An	258	T	F	F	F	F	P	P	P	P	F	G	-	-	N	G	S	G	M	H	C	H	Q	S	L		
St	255	T	T	T	F	F	P	P	P	P	F	G	-	-	N	G	S	G	M	H	C	H	M	S	L		
Tf	253	T	T	T	F	F	P	P	P	P	V	F	G	-	-	N	G	S	G	M	H	V	H	Q	S	L	
Ca	201	T	S	L	D	P	K	P	I	F	G	I	-	-	N	G	S	G	M	H	V	N	M	S	L		
Sc	252	T	F	F	F	F	P	P	P	P	F	G	-	-	N	G	S	G	M	H	V	H	O	S	L		
Bs	230	T	L	F	F	F	P	P	P	L	F	G	-	-	N	G	S	G	M	H	C	N	L	S	L		
Bf	327	T	L	F	F	H	E	K	P	Y	N	G	V	-	-	N	G	S	G	M	H	R	N	N	W	S	L

REGION IV

Hs	317	A	N	R	S	A	S	I	R	I	P
Af	309	A	N	R	G	A	S	I	R	V	G
Bj	290	A	S	O	G	A	S	I	R	V	P
An	341	G	N	R	S	A	S	I	R	I	P
St	338	R	N	R	S	A	S	I	R	I	P
Tf	336	K	N	R	S	A	S	I	R	I	P
Ca	285	K	N	R	T	A	L	I	R	V	P
Sc	337	R	N	R	S	A	A	M	R	I	P
Bs	314	Q	N	R	S	P	L	I	R	I	P

REGION V

Hs	337	F	E	D	R	R	P	S	A	N	C	D	F	L	F	S	V	T	E
Af	329	F	E	D	R	R	P	S	S	N	M	D	P	Y	V	V	T	S	
Bj	311	L	E	D	R	R	P	N	S	Q	G	D	P	V	Q	I	V	R	
An	361	L	E	V	R	C	P	D	A	T	S	N	P	Y	L	A	F	S	
St	357	I	E	V	R	F	F	P	D	P	A	A	N	P	Y	L	C	F	A
Tf	355	I	E	V	R	F	F	P	D	S	T	A	N	P	Y	L	A	F	S
Ca	303	V	E	L	R	C	P	D	P	S	S	N	P	Y	L	V	L	A	
Sc	357	V	E	F	R	A	L	E	D	A	S	G	N	P	Y	L	A	F	S
Bs	332	V	E	V	R	S	V	D	P	A	A	N	P	Y	L	A	L	S	
Bf	471	F	E	F	R	A	A	G	S	S	A	N	C	A	A	A	M	I	

Fig. 4. Comparison of amino acid sequences of five regions of homology of GS enzymes from *Homo sapiens* (Hs) (Gibbs *et al.*, 1987), alfalfa (Af) (Tischer *et al.*, 1986), *Bradyrhizobium japonicum* (Bj) (Carlson & Chelm, 1986), *Anabaena* sp. strain 7120 (An) (Tumer *et al.*, 1983), *Salmonella typhimurium* (St) (Janson *et al.*, 1986), *Thiobacillus ferrooxidans* (Tf) (Rawlings *et al.*, 1987), *Clostridium acetobutylicum* (Ca) (Janssen *et al.*, 1988), *Streptomyces coelicolor* (Sc) (Wray & Fisher, 1988), *Bacillus subtilis* (Bs) (Strauch *et al.*, 1988) and *Bacteroides fragilis* (Bf). The amino acids are identified by the single-letter code and the positions of the first amino acids in each region are indicated. Numbering of residues begins with the start methionine at the N-terminal end. Identical residues are boxed. No region IV was found in *B. fragilis*.

these five regions show far less amino acid similarity although certain conserved amino acids can be identified in four of these five regions.

Region I of the *B. fragilis* GS shows recognizable but relatively poorly conserved amino acid similarity with the other GS enzymes (Fig. 4). The amino acid sequence DGSS at the start of region I is conserved in all the eukaryote and prokaryote GS enzymes. A Ser residue (the homologue of Ser-64 in *Salmonella typhimurium*) is conserved in all GS enzymes except in the *B. fragilis* GS, where it is replaced by a Cys residue (Cys-164 of *B. fragilis*).

In region I the *S. typhimurium* GS has a Trp residue at position 58, which is thought to complete the active site formed between adjacent subunits (Almassy *et al.*, 1986). In the GS from three Gram-positive bacteria, *Bacillus subtilis*, *Clostridium acetobutylicum* and *Streptomyces coelicolor*, the Trp residue is replaced by the functionally similar Phe residue. The *Bacteroides*

Fig. 5. Relative positions of GS regions I to V. The GS enzymes compared are the same as those in Fig. 4. Position 0 is the first amino acid residue of region I. The size and relative positions of regions I to V and the start and end of each GS enzyme are indicated. No region IV was found in *B. fragilis*.

fragilis GS differs from all the other prokaryotic GSs in that the active site Trp in region I is replaced by Val, which shows no functional similarity with Trp (Fig. 4).

In the *Salmonella typhimurium* GS holoenzyme the Trp-58 residue is situated within 0.6 nm of the Tyr residue at position 398 in the carboxy-terminal portion of the adjacent subunit. Tyr-398 has been shown to be the site of adenylylation (Almassy *et al.*, 1986; Shapiro & Stadtman, 1968). Since the Trp-58 of one subunit is situated close to the Tyr-398 of another subunit in the *S. typhimurium* GS, it is suggested that the three-dimensional structure of this region may control the ability of an enzyme to be regulated by adenylylation. The GS enzymes which are subject to adenylylation showed strong similarity among the 18 amino acids adjacent to this Tyr residue (Janssen *et al.*, 1988). *B. fragilis* GS is the first example of a GS from a Gram-negative bacterium which is not regulated by adenylylation (Southern *et al.*, 1987). It is perhaps not surprising, therefore, that the *B. fragilis* GS does not contain either a Trp residue at the relevant position in region I, or an 18-amino-acid sequence containing a Tyr residue which even remotely resembles the *S. typhimurium* adenylylation region.

In both GSI and GSII enzymes, regions II to V are β -strands closely associated with two Mn^{2+} cations of one subunit (Janson *et al.*, 1986). Region II contains four highly conserved amino acids which are not contiguous [E V(X₃₋₄)Q(X₁)E] (Fig. 4). Region III is considered to be the ATP-binding site because the sequence resembles the sequence KXXXXGXXGXGKT found in several ATP-binding proteins (Walker *et al.*, 1982; Tischer *et al.*, 1986). In the *B. fragilis* GS, region III shows the highest degree of conservation: it contains eight amino acid residues which are conserved in all the 10 eukaryote and prokaryote GS enzymes investigated, and an ATP-binding sequence can be identified (Fig. 4). Region IV is thought to be the glutamate-binding site because it is similar to the sequence DRGASIV (amino acid positions 18 to 24) of bovine and chicken glutamine dehydrogenase substrate-binding sites (Moon & Smith, 1972; Rasched *et al.*, 1974; Tischer *et al.*, 1986; Rawlings *et al.*, 1987). The *B. fragilis* GS shows no convincing similarity with the other GS enzymes in region IV. Region V, between positions 471 and 484 of the *B. fragilis* GS, shows relatively high similarity with region V of the *Streptomyces coelicolor* GS between positions 357 and 373. Seven amino acids are conserved and three positions contain amino acid residues which occur in one or more other GS enzymes at the equivalent position (Glu-472, Arg-474 and Asn-481 of *B. fragilis*). These Glu and Arg residues are conserved in all the GS enzymes (Fig. 4).

In Fig. 5 the relative spacings of the GS regions I to V are compared. It is apparent that the relative positions of the best-conserved regions in the *B. fragilis* GS (regions I, II, III and V) are

similar to the positioning of these regions in the other GS enzymes. The start of region I of the GSI subunits is located approximately 50 amino acids from the amino-terminal end, and the end of region V is approximately 100 amino acids from the carboxy-terminal end. In comparison, region I of the *B. fragilis* GS is preceded by 149 amino acids and there is a long carboxy-terminal region of 391 amino acids extending beyond region V. From Fig. 5 it is apparent that in all the GS enzymes the spacing between regions II and III is highly conserved and is approximately 42 amino acid residues.

Conclusions

The GS from the Gram-negative obligate anaerobe *B. fragilis* differs markedly from the GSI and GSII enzymes from other prokaryotes and eukaryotes. Our results demonstrate the very large size of the *B. fragilis* GS subunit, and confirm our previous suggestion that the GS is a hexamer (Southern *et al.*, 1987). Other prokaryote GSI enzymes normally consist of 12 subunits (Streicher & Tyler, 1980; Bhatnagar *et al.*, 1986). The GS from *Clostridium pasteurianum* has been reported to consist of 20 subunits (Krishnan *et al.*, 1986).

Classification of *Bacteroides* species by ribosomal RNA sequence analysis indicated that the genus *Bacteroides* belongs to a distinct assemblage of genera including *Bacteroides*, *Flavobacterium* and *Cytophaga* (Woese, 1987). The taxonomic separation of *Bacteroides* is supported by the structure of the *B. fragilis* GS. It will be interesting to determine whether the GS enzymes of other members of this group are similar to the GS of *B. fragilis*. If this is the case then GS enzymes with large subunits arranged as hexamers should be grouped together to form a third class of GS enzymes (GSIII). Genetic studies in *Agrobacterium* and *Rhizobium* have suggested the possible occurrence of a third GS by the cloning of a gene *glnT* which complements an *E. coli glnA* deletion, allowing growth in the absence of glutamine (Rossbach *et al.*, 1988). However, no enzyme activity has yet been ascribed to the *glnT* gene product.

We are grateful to D. E. Rawlings for supplying GS protein sequences and for useful discussions.

REFERENCES

- AIBA, H., ADHYA, S. & DE CROMBRUGGHE, B. (1981). Evidence for two functional *gal* promoters in intact *Escherichia coli* cells. *Journal of Biological Chemistry* **256**, 11905–11910.
- ALMASSY, R. J., JANSON, C. A., HAMLIN, R., XUONG, N.-H. & EISENBERG, D. (1986). Novel subunit-subunit interactions in the structure of glutamine synthetase. *Nature, London* **323**, 304–309.
- AMES, G. F.-L. & NIKAI, K. (1985). Nitrogen regulation in *Salmonella typhimurium*. Identification of an *ntrC* protein-binding site and definition of a consensus binding sequence. *EMBO Journal* **4**, 539–547.
- BHATNAGAR, L., ZEIKUS, J. G. & AUBERT, J.-P. (1986). Purification and characterization of glutamine synthetase from the archaeobacterium *Methanobacterium ivanovi*. *Journal of Bacteriology* **165**, 638–643.
- BOZOUKLIAN, H. & ELMERICH, C. (1986). Nucleotide sequence of the *Azospirillum brasilense* Sp7 glutamine synthetase structural gene. *Biochimie* **68**, 1181–1187.
- CARLSON, T. A. & CHELM, B. K. (1986). Apparent eukaryotic origin of glutamine synthetase II from the bacterium *Bradyrhizobium japonicum*. *Nature, London* **322**, 568–570.
- COLOMBO, G. & VILLAFRANCA, J. J. (1986). Amino acid sequence of *Escherichia coli* glutamine synthetase deduced from the DNA nucleotide sequence. *Journal of Biological Chemistry* **261**, 10587–10591.
- GEBHARDT, C., OLIVER, J. E., FORDE, B. G., SAARELAINE, R. & MIFLIN, B. (1986). Primary structure and differential expression of glutamine synthetase genes in nodules, roots and leaves of *Phaseolus vulgaris*. *EMBO Journal* **5**, 1429–1435.
- GIBBS, C. S., CAMPBELL, K. E. & WILSON, R. H. (1987). Sequence of a human glutamine synthetase cDNA. *Nucleic Acids Research* **15**, 6293.
- HAYWARD, B. E., HUSSAIN, A., WILSON, R. H., LYONS, A., WOODCOCK, V., MCINTOSH, B. & HARRIS, T. J. R. (1986). The cloning and nucleotide sequence of cDNA for an amplified glutamine synthetase gene from the Chinese hamster. *Nucleic Acids Research* **14**, 999–1008.
- HENIKOFF, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28**, 351–359.
- HOLDEMAN, L. V. & MOORE, W. E. C. (1972). *Anaerobe Laboratory Manual*. Blacksburg, USA: Virginia Polytechnic Institute and State University.
- ISH-HOROWICZ, D. & BURKE, J. F. (1981). Rapid and efficient cosmid cloning. *Nucleic Acids Research* **9**, 2989–2998.
- JANSON, C. A., KAYNE, P. S., ALMASSY, R. J., GRUNSTEIN, M. & EISENBERG, D. (1986). Sequence of glutamine synthetase from *Salmonella typhimurium* and implications for the protein structure. *Gene* **46**, 297–300.
- JANSSEN, P. J., JONES, W. A., JONES, D. T. & WOODS, D. R. (1988). Molecular analysis and regulation of the *glnA* gene of the Gram-positive anaerobe

- Clostridium acetobutylicum*. *Journal of Bacteriology* **170**, 400–408.
- KRISHNAN, I. S., SINGHAL, R. K. & DUA, R. D. (1986). Purification and characterization of glutamine synthetase from *Clostridium pasteurianum*. *Biochemistry* **25**, 1589–1599.
- MILLER, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MOON, K. & SMITH, E. L. (1973). Sequences of bovine liver glutamate dehydrogenase. VIII. Peptides produced by specific chemical cleavages, the complete sequence of the protein. *Journal of Biological Chemistry* **248**, 3082–3088.
- MOSSIE, K. G., JONES, D. T., ROBB, F. T. & WOODS, D. R. (1979). Characterization and mode of action of a bacteriocin produced by a *Bacteroides fragilis* strain. *Antimicrobial Agents and Chemotherapy* **16**, 724–730.
- PRUSINER, S. & STADTMAN, E. R. (1973). *The Enzymes of Glutamine Metabolism*. New York: Academic Press.
- RASCHED, I., JORNVAL, H. & SUND, H. (1974). Studies on glutamate dehydrogenase: identification of an amino group involved in the substrate binding. *European Journal of Biochemistry* **41**, 603–606.
- RAWLINGS, D. E., JONES, W. A., O'NEILL, E. G. & WOODS, D. R. (1987). Nucleotide sequence of the glutamine synthetase gene and its controlling region from the acidophilic autotroph *Thiobacillus ferrooxidans*. *Gene* **53**, 211–217.
- ROSSBACH, S., SCHELL, J. & DE BRUIJN, F. J. (1988). Cloning and analysis of *Agrobacterium tumefaciens* C58 loci involved in glutamine biosynthesis: neither the *glnA* (GSI) nor the *glnII* (GSII) gene plays a special role in virulence. *Molecular and General Genetics* **212**, 38–47.
- SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463–5467.
- SHAPIRO, B. M. & STADTMAN, E. R. (1968). 5'-Adenylyl-O-tyrosine. The novel phosphodiester of adenylylated glutamine synthetase from *Escherichia coli*. *Journal of Biological Chemistry* **243**, 3769–3771.
- SHINE, J. & DALGARNO, L. (1976). Determinant of cistron specificity in bacterial ribosomes. *Nature, London* **254**, 34–38.
- SOUTHERN, J. A., PARKER, J. R. & WOODS, D. R. (1986). Expression and purification of glutamine synthetase cloned from *Bacteroides fragilis*. *Journal of General Microbiology* **132**, 2827–2835.
- SOUTHERN, J. A., PARKER, J. R. & WOODS, D. R. (1987). Novel structure, properties and inactivation of glutamine synthetase cloned from *Bacteroides fragilis*. *Journal of General Microbiology* **133**, 2437–2446.
- STRAUCH, M. A., ARONSON, A. I., BROWN, S. W., SCHREIER, H. J. & SONENSHEIN, A. L. (1988). Sequence of the *Bacillus subtilis* glutamine synthetase gene region. *Gene* **71**, 257–265.
- STREICHER, S. L. & TYLER, B. (1980). Purification of glutamine synthetase from a variety of bacteria. *Journal of Bacteriology* **142**, 69–78.
- TISCHER, E., DAS SARMA, S. & GOODMAN, H. M. (1986). Nucleotide sequence of an alfalfa glutamine synthetase gene. *Molecular and General Genetics* **203**, 221–229.
- TUMER, N. E., ROBINSON, S. J. & HASELKORN, R. (1983). Different promoters for the *Anabaena* glutamine synthetase gene during growth using molecular or fixed nitrogen. *Nature, London* **306**, 337–342.
- WALKER, J. E., SARASTE, M., RUNWICK, M. J. & GAY, N. J. (1982). Distantly related sequences in the α - and β -subunits of ATP synthetase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO Journal* **1**, 945–951.
- WOESE, C. R. (1987). Bacterial evolution. *Microbiological Reviews* **51**, 221–271.
- WRAY, L. V., JR & FISHER, S. H. (1988). Cloning and nucleotide sequence of the *Streptomyces coelicolor* gene encoding glutamine synthetase. *Gene* **71**, 247–256.
- ZABEAU, M. & STANLEY, K. (1982). Enhanced expression of *cro*- β -galactoside fusion proteins under the control of the P_r promoter of bacteriophage λ . *EMBO Journal* **1**, 1217–1224.