The *glnA* gene of the extremely thermophilic eubacterium *Thermotoga maritima*: cloning, primary structure, and expression in *Escherichia coli*

ANNA M. SANANGELANTONI,¹ GIUSEPPE FORLANI,¹ FRANCO AMBROSELLI,¹ PIERO CAMMARANO² and ORSOLA TIBONI^{1*}

¹Dipartimento di Genetica e Microbiologia 'A. Buzzati-Traverso', Università di Pavia, via Abbiategrasso 207, 27100 Pavia, Italy

²Dipartimento di Biopatologia Umana, Sezione Biologia Cellulare, Università di Roma 'La Sapienza', Policlinico Umberto I°, I-00161, Roma, Italy

(Received 10 September 1991; accepted 21 October 1991)

The structural gene (*glnA*) encoding the glutamine synthetase (GS) of the extremely thermophilic eubacterium *Thermotoga maritima* has been cloned on a 6.0 kb *Hind*III DNA fragment. Sequencing of the region containing the *glnA* gene (1444 bp) showed an ORF encoding a polypeptide (439 residues) with an estimated mass of 50088 Da, which shared significant homology with the GSI sequences of other Bacteria (*Escherichia coli*, *Bacillus subtilis*) and Archaea (*Pyrococcus woesei*, *Sulfolobus solfataricus*). The *T. maritima glnA* gene was expressed in *E. coli*, as shown by the ability to complement a *glnA* lesion in the glutamine-auxotrophic strain ET8051. The recombinant GS has been partially characterized with respect to the temperature dependence of enzyme activity, molecular mass and mode of regulation. The molecular mass of the *Thermotoga* GS (590000 Da), estimated by gel filtration, was compatible with a dodecameric composition for the holoenzyme, as expected for a glutamine synthetase of the GSI type. Comparison of the amino acid sequence of *T. maritima* GS with those from thermophilic and mesophilic micro-organisms failed to detect any obvious features directly related to thermal stability.

Introduction

With the discovery of extremely thermophilic and hyperthermophilic Archaea and Bacteria, the upper limit of temperature compatible with life has been raised above 100 °C (Huber *et al.*, 1986; Zillig *et al.*, 1990; Pledger & Baross, 1991). The strategies whereby proteins from these micro-organisms remain stable at temperatures equal to or higher than the boiling point of water are still largely undefined. It is also unclear how thermophilic enzymes succeed in combining the structural rigidity required for stabilization with the flexibility required for catalytic function. Because proteins from thermophilic micro-organisms retain their heat-resistance upon expression in mesophilic hosts (Love & Streiff, 1987; Love *et al.*, 1988; Tiboni *et al.*, 1989), their

ability to withstand high temperatures appears to be determined only by features of primary structure. Therefore, sequence comparison of homologous proteins from mesophilic and thermophilic organisms may enable sequence traits critical for thermal stabilization to be identified (Fabry *et al.*, 1989; Schultes *et al.*, 1990).

In this work on the extremely thermophilic eubacterium *Thermotoga maritima*, attention has been focused on the key enzyme for ammonia assimilation glutamine synthetase (GS). This protein has been extensively investigated at the biochemical and molecular levels in a wide range of phylogenetically diverse bacterial/archaeal and eukaryotic species (Streicher & Tyler, 1980; Bhatnagar *et al.*, 1986; Cullimore & Bennet, 1988). In both Bacteria and Archaea, GS is an oligomeric enzyme, termed GSI, consisting of twelve identical subunits each with a molecular mass of 50–55 kDa. The only known exception is the anaerobic bacterium *Bacteroides fragilis*, whose GS is composed of six subunits each of 83 kDa (Hill *et al.*, 1989). The eukaryotic GS, termed GSII, differs from the bacterial and archaeal enzymes in comprising eight subunits, each of 45–48 kDa, whose primary sequence has little similarity to those of the

* Author for correspondence. Tel. (0382) 391578; fax (0382) 528496.

Abbreviations: EF, elongation factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GS, glutamine synthase; SVP, snake venom phosphodiesterase.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number X60160.

bacterial and archaeal enzymes. Interestingly, members of *Rhizobium* and *Streptomyces* groups harbour both GSI (bacterial) and GSII (eukaryotic) enzymes (Carlson & Chelm, 1986; Shatters & Kahn, 1989; Behrmann *et al.*, 1990; Kumada *et al.*, 1990). Although bacterial GSs differ substantially from their eukaryotic homologues, it is possible to identify five highly conserved regions, all of which are associated with putative active sites (Almassy *et al.*, 1986; Rawlings *et al.*, 1987).

Glutamine synthetases from different organisms also differ in their mode of regulation. The enzyme synthesizes glutamine from ammonia and glutamate, which in turn is the nitrogen source for the biosynthesis of a range of metabolites, some of which act as feedback inhibitors of GS activity (Deuel & Prusiner, 1974). In certain bacteria (enteric bacteria, *Streptomyces*), however, the GS activity is also regulated by the adenylation of a tyrosyl residue (position 397 of the *Escherichia coli* GS) of each of the component subunits (Reitzer & Magasanik, 1987; Streicher & Tyler, 1981; Fisher & Wray, 1989). This mechanism is not present in eukaryotes, whose GS lacks the C-terminal region including the adenylation site.

Here we report the isolation, sequencing and expression in *E. coli* of the *T. maritima* gene (*glnA*) encoding glutamine synthetase. The derived amino acid sequence has been compared with the equivalent sequences of other mesophilic and thermophilic micro-organisms. The protein encoded by the *T. maritima* gene has been partially characterized with regard to temperature dependence of catalytic rates, molecular mass and the existence of an adenylation-deadenylation mechanism.

Methods

Bacterial strains and plasmids. *T. maritima* MSB8 (DSM 3109) cells, grown as described by Huber *et al.* (1986), were kindly supplied by R. Huber. Plasmid pSpC2 (Riccardi *et al.*, 1985), containing the *glnA* gene of *Spirulina platensis*, was used to prepare the heterologous probe and was also used as the control in complementation experiments. Plasmids pBR322 (Bolivar *et al.*, 1977) and pUC19 (Vieira & Messing, 1982) were used as the vectors and *E. coli* HB101 as the host. *E. coli* ET8051 [$\Delta(rha-glnA)$ *hutC_k*, *rbs nal^r*] kindly provided by D. MacNeil, Merck, Sharp & Dohme Research Laboratories, Rahway, USA, was used in complementation experiments.

Recombinant DNA techniques. Chromosomal DNA from *T. maritima* was prepared as described by Blin & Stafford (1976). Plasmid DNA (large and small scale) was isolated and purified according to Maniatis *et al.* (1982). DNA fragments were recovered from low-melting agarose gels as reported by Higuchi *et al.* (1981). Transformation experiments were carried out as described by Dagert & Ehrlich (1979). Hybridization experiments (Southern, 1975) were performed at 37 °C in the presence of 35% formamide.

DNA sequencing. The inserts of plasmids pTM15 and pTM16 were digested with different restriction enzymes, and fragments ranging from 100 to 650 bp were subcloned in the polylinker cloning sites of

pUC19. Sequencing of both DNA strands was done by the dideoxy chain-termination method of Sanger *et al.* (1977) with overlapping templates. The DNA was radiolabelled with [³⁵S]dATP (deoxyadenosine 5'- α -[³⁵S]thiotriphosphate) (>1000 Ci mmol⁻¹) (>3.7 $\times$ 10¹³ Bq mmol⁻¹) and primed as specified by the manufacturer using a T7 Sequencing kit (Pharmacia).

Analysis of the DNA sequence was performed using a Macintosh IICx computer and the DNA Strider 1.0 program (Marck, 1988). Alignment of protein sequences was performed using an IBM PC and the PRTALN and DM subroutines of the NIH-MBUG program (Wilbur & Lipman, 1983).

Expression studies. Plasmids pTM16, pSpC2 and pBR322 were used to transform the *E. coli* strain ET8051. Transformants, selected on LB plates supplemented with 50 μ g ml⁻¹ ampicillin and 0.2% glutamine, were replicated on plates of M9 minimal medium and M9 minimal medium supplemented with 0.2% glutamine and incubated at 37 °C for 2–4 d. For biochemical analysis, cells were grown at 37 °C in M9 minimal medium with constant shaking to OD₆₅₀ $\approx$ 0.7. The cells were harvested by centrifugation and washed once with extraction buffer (100 mM-Tris/HCl, pH 7.4; 6 mM- β -mercaptoethanol).

Enzyme assays. Cell-free extracts of *E. coli* and *T. maritima* were prepared at 4 °C by grinding (in a mortar) 1 g of cells with 2 g of alumina powder (Sigma). The paste was resuspended in 5 ml of extraction buffer and centrifuged at 20000 g for 30 min. The supernatant (crude extract) was then desalted through a column (1.6 $\times$ 10 cm) of Bio-Gel P6DG (Biorad) equilibrated with the extraction buffer. For GS transferase activity assays, crude extracts were buffer-exchanged by using a column equilibrated with 50 mM-imidazole/HCl buffer (pH 7.4) containing 1 mM- β -mercaptoethanol. Glutamine synthetase was assayed for both hemibiosynthetic and transferase activities as described by Haizer & Moses (1978) and Stadtman *et al.* (1979). The synthetic assay was used routinely, because it provides a better estimate of the catalytic rate of the enzyme under physiological conditions; appropriate controls in the presence of 100 mM-proline were performed in order to avoid interference by γ -glutamyl kinase activity. One unit (U) of GS activity is defined as the amount of enzyme catalysing the production of 1 nmol s⁻¹ of γ -glutamylhydroxamate (nkat). The pH dependence of the transferase activity was determined in the presence of 200 mM-imidazole buffer over the pH range 6.0–8.5. The occurrence of enzyme adenylation was estimated in imidazole buffer assay mixture (Stadtman *et al.*, 1979) by monitoring the shift in metal ion specificity after incubation of the enzyme with snake venom phosphodiesterase (type I from *Crotalus adamanteus*) (Sigma) (Shapiro *et al.* 1967). Protein concentrations were determined as described by Bradford (1976) using bovine serum albumin as the standard.

Relative molecular mass determination. Native molecular mass was estimated by gel filtration using a Sephacryl S300 HR (Pharmacia) column (1.6 $\times$ 82 cm) calibrated with the appropriate molecular mass standards. The column was equilibrated and eluted with ice-cold extraction buffer. Samples (2 ml) were applied, and elution was carried out at a flow-rate of 12 ml h⁻¹, while collecting 1 ml fractions.

Results

Cloning of the *T. maritima glnA* gene

To identify the *T. maritima glnA* gene, genomic DNA digested with different restriction enzymes was probed with the *glnA* gene from *S. platensis*. Among the digestions exhibiting a single hybridization signal with

Fig. 1. Endonuclease restriction map of pTM15 and pTM16, and sequencing strategy for the *T. maritima* *glnA* structural gene. Arrows indicate the directions and the extent of the DNA region sequenced.

the cyanobacterial probe, a 3.5 kb *EcoRI* fragment was selected for study.

A size-selected *EcoRI* genomic library of *T. maritima* DNA (3.0–4.3 kb *EcoRI* fragments) was constructed in pBR322 using *E. coli* HB101 as the host. Since the signal produced by the *T. maritima* *glnA* gene was as weak as that generated by the *glnA* gene of the host, it was impossible to carry out colony hybridizations. Thus, recombinant plasmids harbouring sequences homologous to the probe were selected by analysing the inserts. Plasmid DNA, prepared from 30 pools of 18 recombinant clones each, was digested with *EcoRI*. The resultant fragments were separated by gel electrophoresis, and hybridized with the *S. platensis* *glnA* probe. The individual transformants from two positive pools were analysed for plasmid content, and a recombinant plasmid, containing a 3.5 kb *EcoRI* fragment (designated pTM15), was isolated. The restriction map of pTM15 was determined and the gene region was localized to a 1.0 kb *HaeIII*/*EcoRI* fragment that included the unique *EcoRV* site (Fig. 1). The *HaeIII*/*EcoRI* fragment from pTM15 was subcloned into pUC19 and sequenced, following the strategy outlined in Fig. 1.

Sequence analysis revealed the presence of an incomplete ORF encoding a polypeptide of 328 amino acid residues highly homologous to the N-terminal portion of eubacterial GSs. Thus, in order to localize the entire *glnA* gene, genomic DNA of *T. maritima* was digested with a range of restriction enzymes, and probed with the *HaeIII*/*EcoRI* fragment of pTM15 and the *HindIII* digestion, which showed hybridization with a 6.0 kb fragment, was selected.

Colony hybridization of the partial *HindIII* genomic library allowed the isolation of two recombinant plasmids with an insert homologous to the probe. The restriction map of one of these plasmids, designated pTM16, demonstrated the presence, within the cloned fragment, of the entire insert of pTM15 (Fig. 1).

Nucleotide sequence of the *T. maritima* *glnA* structural gene

The complete nucleotide sequence of the 1444 bp *T. maritima* DNA fragment containing the *glnA* gene was determined following the strategy outlined in Fig. 1. Analysis of the sequence (Fig. 2) revealed the presence of

1	GGCCAAACAACATTCATTTTCATGTCCTGGGATATTGATTATACACAAATGATTTTATGTTCAAGAGGAGGTGTGTGTT	78
	SD	
79	ATG ACA ATT GAG ACC ATC AAA AGA ATC ATC GAA GAG GAG AAT GTT CGA TTC ATC AGG CTG Met thr ile glu thr ile lys arg ile ile glu glu glu asn val arg phe ile arg leu	138
139	CAA TTC ACC GAC ATC AAC GGG ACC TTG AAG AAT TTA GAG ATA ACG CCG GAT GTT TTT CTC gln phe thr asp ile asn gly thr leu lys asn leu glu ile thr pro asp val phe leu	198
199	GAA TCC TGG GAG GAC GGA ATC ATG TTC GAC GGT TCA TCC ATC GAG GGT TTT GTG AGA ATC glu ser trp glu asp gly ile met phe asp gly ser ser ile glu gly phe val arg ile	258
259	GAA GAG TCT GAT ATG TAC CTC AAG CCC GTC CTC GAC ACC TTC GCC GTT CTT CCG TGG ACG glu glu ser asp met tyr leu lys pro val leu asp thr phe ala val leu pro trp thr	318
319	GTC GAT GGT GCA AAG AGT GCA AGA GTC ATC TGT GAC GTT TAC ACT CCA GAT GGT AAA CCC val asp gly ala lys ser ala arg val ile cys asp val tyr thr pro asp gly lys pro	378
379	TTT GAG GGA GAT CCC CGT TAC AGA CTA AGA AGA ATG ATG GAA AAA GCA GAA CAG CTG GGT phe glu gly asp pro arg tyr arg leu arg arg met met glu lys ala glu gln leu gly	438
439	TAC ACA CCG TAT GCG GGT CCC GAG ATG GAA TTT TTC ATC CTT CCC ATC AAC GAA AAA GGA tyr thr pro tyr ala gly pro glu met glu phe phe ile leu pro ile asn glu lys gly	498
499	GAA CCT GTT CCT GAG TTT CTT GAC CAT GGA GGA TAT TTC GAT CTT CTG CCG CTC AGT AAA glu pro val pro glu phe leu asp his gly gly tyr phe asp leu leu pro leu ser lys	558
559	GTC GAG GAG ATC AGA AGG GAC ATC GCG ATA GCT CTC GAA AAG ATG GGG ATC ACT GTT GAA val glu glu ile arg arg asp ile ala ile ala leu glu lys met gly ile thr val glu	618
619	GCA ACG CAC CAC GAA GTT GCT CCC TCA CAG CAC GAG GTG GAC TTC AGG TAC GAC ACT TTC ala thr his his glu val ala pro ser gln his glu val asp phe arg tyr asp thr phe	678
679	CTG AGA ACC GGC GAC AAT GCC CAG ACC GTG AAA CTC GTC ATC AAA ACC ATG GCA ATC TTC leu arg thr gly asp asn ala gln thr val lys leu val ile lys thr met ala ile phe	738
739	CAC GGA TAT CAT GCA ACC TTC ATG CCC AAA CCC TTC TAC GGT GTG AAC GGA TCG GGA ATG his gly tyr his ala thr phe met pro lys pro phe tyr gly val asn gly ser gly met	798
799	CAT GTC CAC ATG AGT CTT TTC AGG GGT GAC AAA AAT GCC TTC TAC GAT CCC GAC GAT CCT his val his met ser leu phe arg gly asp lys asn ala phe tyr asp pro asp asp pro	858
859	CTT GGG CTT TCT AAA GAA CTC AGA TAC TTC GTT GGT GGT ATA TTG AAG CAC GCC AAA CGT leu gly leu ser lys glu leu arg tyr phe val gly gly ile leu lys his ala lys arg	918
919	CTC GCA GCC GTC ACA AAT CCC ACG ATA AAC AGC TAC AAG AGA CTC GTT CCC GGT TAC GAA leu ala ala val thr asn pro thr ile asn ser tyr lys arg leu val pro gly tyr glu	978
979	GCG CCC GTT TAC ATC TCC TGG TCA GTT GGA AAC AGA AGC GCC CTC ATC AGA ATT CCA AAG ala pro val tyr ile ser trp ser val gly asn arg ser ala leu ile arg ile pro lys	1038
1039	GCA AGA GGA AAA GCG ACA AGA TTG GAG TAC AGA TCA CCC GAT CCT ACC TGT AAC ATC TAT ala arg gly lys ala thr arg leu glu tyr arg ser pro asp pro thr cys asn ile tyr	1098
1099	CTT GCC TTC GCG GCT ATC CTC GCG GCA GGT CTG GAC GGT ATC ATC AAC AAA ATC GAG CCT leu ala phe ala ala ile leu ala ala gly leu asp gly ile ile asn lys ile glu pro	1158
1159	CCC GCG CCG GTT GAA GAA AAC ATT TAC CAC ATG ACG TCT GAG AGA AGG GAA GAA GTC AAC pro ala pro val glu glu asn ile tyr his met thr ser glu arg arg glu glu leu asn	1218
1219	ATA GAA TCG CTT CCA GGA TCA CTG AAA GAG GCT GTC GAA GAA CTC AAG AAA GAC GAT GTT ile glu ser leu pro gly ser leu lys glu ala val glu glu leu lys lys asp asp val	1278
1279	ATA ATA GAT GCA CTG GGA GAA CAT ATA TTC GAA AAA TTT GTG GAG GCT GCC GAA AAG GAT ile ile asp ala leu gly glu his ile phe glu lys phe val glu ala ala glu lys asp	1338
1339	TGG AAG GAA TTC AGC ACC TAC GTT ACA AAC TGG GAA CTG CAG CTC TAC TTG TAT CTC TGA trp lys glu phe ser thr tyr val thr asn trp glu leu gln arg tyr leu tyr leu TER	1398
1399	TCGTAAGTGTTTTCGCGGAATCCTCGAAACAGAGAGGATAGATCT	1444

an ORF of 1320 bp preceded by a ribosome binding site (Shine & Dalgarno, 1976) (GAGGAGG) 7 bp upstream from the putative start codon. The derived polypeptide (439 amino acid residues, with a predicted mass of 50088 Da) is formally similar to GS subunits from *Bacillus subtilis* (Nakano *et al.*, 1989), *Clostridium acetobutylicum* (Janssen *et al.*, 1988) and *Methanococcus voltae* (Possot *et al.*, 1989), but slightly shorter than GS subunits from *E. coli* (Colombo & Villafranca, 1986) and other Bacteria (Toukdarian *et al.*, 1990). The identity of the 1320 bp ORF with the *T. maritima* *glnA* gene was unambiguously established by the alignment of the derived polypeptide with the GS proteins of two extremely thermophilic archaeotes (*Pyrococcus woesei* and *Sulfolobus solfataricus*) and with those of the mesophilic bacteria *B. subtilis* and *E. coli* (Fig. 3). The *T. maritima* GS exhibits considerable sequence similarity with those of *P. woesei* (48.3%) and *B. subtilis* (53.7%) and significant similarity with those of *E. coli* (32.8%) and *S. solfataricus* (37.5%). Fig. 3 also shows that the five regions (marked by asterisks) associated with active sites of the enzyme (Rawlings *et al.*, 1987) are rather stringently conserved.

The *T. maritima* enzyme shares with *P. woesei*, *M. voltae*, *C. acetobutylicum* and *B. subtilis* a deletion of 26 amino acids from positions 145 to 171. This region has been identified as a large protease-sensitive loop (Almassy *et al.*, 1986). No thermophilic-specific features are detectable that distinguish the *T. maritima*, *S. solfataricus* and *P. woesei* GS sequences from their mesophilic counterparts, except for the presence of a tyrosine residue at position 372, which is replaced by a valine in the mesophilic sequences. The relevance of this substitution with respect to thermal stability, however, is uncertain.

The amino acid sequence around Tyr residue 411 (Fig. 3) is of particular interest, because this residue has been identified in *E. coli* as the site of GS adenylation (Shapiro & Stadtman, 1968). The region flanking this site (18 amino acids) is highly conserved in all GSs whose activity is regulated by adenylation (Janssen *et al.*, 1988). Although *T. maritima* GS has a Tyr residue at the correct position, only 4 out of the 18 nearby residues are identical to those present in *E. coli*, suggesting that the enzyme activity is not modulated by an adenylation-deadenylation mechanism.

Expression of the *T. maritima* *glnA* gene in *E. coli*

The *E. coli* glutamine auxotroph ET8051 carrying a deletion for the glutamine synthetase gene was transformed with the recombinant plasmids pTM16 and

Table 1. GS specific activities in cell-free extracts from recombinant strains

GS activity is expressed as nkat (mg protein)⁻¹. Results are means of at least three independent determinations.

<i>E. coli</i> strain	<i>glnA</i> allele	Synthetic assay			Transferase assay (37 °C)
		37 °C	70 °C	90 °C	
ET8051 pBR322	none	0.00	0.00	0.00	0.00
ET8051 pTM16	<i>T. maritima</i>	1.24	16.20	38.13	53.20
ET8051 pTM16*	<i>T. maritima</i>	0.84	ND	ND	35.80
ET8051 pSpC2	<i>S. platensis</i>	2.50	17.67	0.00	222.30

ND, not determined.

* Culture grown as described in Methods in minimal medium containing 0.2% glutamine.

pSpC2 carrying, respectively, the *glnA* gene of *T. maritima* and of *S. platensis* (a mesophilic bacterium) and with the vector pBR322 as control. Transformants selected on LB medium containing ampicillin were replicated on M9 minimal medium containing 0.1% NH₄Cl as the sole nitrogen source. After 48 h incubation at 37 °C, only the transformants harbouring pSpC2 were able to grow on minimal medium; transformants harbouring the plasmid pTM16 exhibited appreciable growth only after four days. Under identical conditions, no growth was observed of transformants harbouring the vector pBR322 alone.

These results indicate that the *glnA* gene of *T. maritima* is able to complement the *glnA* lesion present in the *E. coli* ET8051 mutant, although less efficiently than the *glnA* gene from *S. platensis*. The long time period (96 h) required for the growth of cells complemented by pTM16 might reflect low activity of the thermophilic *T. maritima* GS enzyme at the host growth temperature (37 °C).

To investigate this point, GS activities were determined at increasing temperatures by the hemibiosynthetic or the transferase assays, using cell extracts prepared from recombinant strains grown in the presence or absence of 0.2% glutamine (Table 1). The former assay estimates the rate of glutamate conversion to γ -glutamyl hydroxamate when ammonia is replaced by hydroxylamine, whereas the latter evaluates the reverse reaction from glutamine and ADP. As Table 1 shows, at 37 °C extracts from *E. coli* transformed with plasmid pTM16 exhibit considerably less GS activity than comparable extracts prepared from *E. coli* cells transformed with plasmids carrying the *S. platensis* *glnA* gene. Conversely, at a temperature (90 °C) close to the physiological optimum for *T. maritima* growth, only the extracts from *E. coli* cells carrying the pTM16 exhibit GS activity.

Fig. 2. Complete nucleotide sequence of the *T. maritima* *glnA* gene. The deduced amino acid sequence is shown below the DNA sequence. The Shine-Dalgarno (SD) sequence is underlined.

Fig. 3. Alignment of the *T. maritima* (Tm) GS amino acid sequence with the sequence of GS enzymes from: Pw, *P. woesei* (our laboratory, in preparation); Ss, *S. solfataricus* (Sanangelantoni *et al.*, 1990); Bs, *B. subtilis* (Nakano *et al.*, 1989); and Ec, *E. coli* (Colombo & Villafranca, 1986). The five conserved regions are underlined with asterisks. The Tyr residue adenylated in *E. coli* is indicated by an asterisk. † indicates the Tyr residue present in the thermophiles and substituted in the mesophiles by a Val residue.

Fig. 4. Native molecular mass determination of *T. maritima* GS. Crude extracts of both *T. maritima* and *E. coli* harbouring the plasmid pTM16 were separated by gel filtration, and eluted fractions were assayed for GS synthetic activity. The column was calibrated using the following standards: bovine thyroid thyroglobulin (669 kDa), horse spleen ferritin (440 kDa), bovine liver catalase (232 kDa), rabbit muscle aldolase (158 kDa), bovine serum albumin (67 kDa), hen egg ovalbumin (43 kDa), bovine pancreas chymotrypsinogen (25 kDa) and bovine pancreas ribonuclease A (13.7 kDa).

The results in Table 1 also show that the GS activity of *E. coli* ET8051 cells complemented with pTM16 is not significantly affected by the presence or absence of exogenous glutamine during cell growth. Since the host strain also carries a deletion for genes (*ntrB* and *ntrC*) involved in the transcriptional regulation of the *glnA* gene (Reitzer & Magasanik, 1987), the insensitivity of GS to glutamine levels during cell growth indicates that no genes equivalent to *ntr* are present on the cloned fragment.

Molecular mass and temperature dependence of *T. maritima* GS expressed in *E. coli*

To determine whether the thermophilic GS subunits are correctly assembled during their synthesis in the mesophile host (*E. coli*), the molecular mass of the recombinant holoenzyme was compared with that of authentic *T. maritima* enzyme. To this aim, crude extracts prepared from *E. coli* cells harbouring the recombinant plasmid (pTM16) and from *T. maritima* cells were chromatographed on a gel filtration column calibrated previously with appropriate molecular mass standards, and GS was identified by monitoring the synthetic activity of the effluent fractions. Both the recombinant and the authentic enzymes eluted as a single peak, with a retention volume indicating a molecular mass of about 590 000 Da (Fig. 4). Since sequence data indicated a

subunit of 50088 Da, the molecular mass of the holoenzyme is consistent with a dodecameric composition of the native protein.

Because the recombinant GS is synthesized at a temperature 50 °C lower than the physiological optimum for *T. maritima* growth, it seemed possible that its folding pattern might differ more or less substantially from that of authentic *T. maritima* GS synthesized at 87 °C. This has been shown to occur in the case of *T. maritima* elongation factor Tu (EF-Tu) expressed in *E. coli*, which requires thermal activation for GDP-binding activity (Tiboni *et al.*, 1989). This possibility is ruled out by the results in Fig. 5, which illustrates the temperature dependence of the recombinant GS activity and the corresponding Arrhenius plot. As the graphs show, the velocity of the forward reaction increases with a Q_{10} of about 2 over a broad temperature range (Fig. 5a). Accordingly, the Arrhenius plot does not exhibit deviations from linearity over the range 30–75 °C (Fig. 5b). This is consistent with no activation energy being required to overcome conformational barriers that hinder the achievement of the physiological folding pattern. The results in Fig. 5 also show that the *T. maritima* enzyme is considerably more stable than the mesophilic enzyme. In fact, no rapid inactivation of the enzyme occurs at temperatures above 90 °C (Fig. 5a). In contrast, the GSs from *E. coli* and *S. platensis* (expressed in *E. coli*) are completely inactivated at 75–80 °C (data not shown). From the Arrhenius plot in Fig. 5(b), the activation energy of the reaction was estimated to be 65.8 kJ mol⁻¹, a value significantly higher than that calculated for the cyanobacterial GS expressed in *E. coli* (49.8 kJ mol⁻¹, data not shown).

Effect of snake venom phosphodiesterase (SVP) treatment on GS transferase activity

Finally, the possible occurrence of an adenylation-deadenylation mechanism modulating the *T. maritima* GS activity was investigated. In bacterial systems, Mg²⁺ ions are known to inhibit the transferase activity of the adenylylated subunits of the enzyme (Stadtman *et al.*, 1979). Since SVP removes adenylic residues covalently bonded to the GS subunits, pretreatment of an adenylylated enzyme with SVP is expected to relieve Mg²⁺ inhibition of GS transferase activity (Shapiro *et al.*, 1967). As Table 2 shows, Mg²⁺ ions did, in fact, exert a strong inhibition on the *T. maritima* GS activity in extracts of *E. coli* cells grown in the presence of ammonia, as might have been expected if the *T. maritima* enzyme was adenylylated. However, treatment of the enzyme with SVP neither increased GS activity in the presence of Mg²⁺ nor caused a significant shift in the pH-activity relationship, which still exhibited a broad

Fig. 5. Effect of temperature on forward reaction of *T. maritima* glutamine synthetase. (a) GS hemibiosynthetic activity versus temperature; (b) Arrhenius plot.

Table 2. Effect of Mg^{2+} ions on GS transferase activity before and after SVP treatment

Cultures were grown in excess nitrogen. GS activity is expressed as nkat (mg protein)⁻¹. Results are means of two independent experiments.

<i>E. coli</i> strain	<i>glnA</i> allele	Before SVP treatment			After Mg^{2+} (60 mM)
		Mn^{2+} only	Mg^{2+} (1 mM)	Mg^{2+} (60 mM)	
HB101	<i>E. coli</i>	6.4	ND	12.0%	36.0%
ET8051 pTM16	<i>T. maritima</i>	53.2	34%	3.1%	2.5%
ET8051 pSpC2	<i>S. platensis</i>	222.0	82%	41.0%	42.0%

ND, not determined.

optimum around pH 7.0 (data not presented). These results seem to indicate that the *T. maritima* GS is not regulated by an adenylation-deadenylation system. Similar data were obtained for the *S. platensis* GS (Table 2). This conclusion is also supported by the low degree of conservation of the residues surrounding the *T. maritima* tyrosine residue which in *E. coli* acts as the adenylation site (Fig. 3).

Discussion

The expression of genes encoding proteins from extreme thermophilic micro-organisms in a mesophilic host offers two significant advantages. Firstly, it overcomes the problem of low biomass yield usual with thermophilic cultures. Secondly, it allows the partial purification, in a

single step, of the heterologous heat-stable proteins by selective thermal denaturation of the mesophilic host cell proteins (Tiboni *et al.*, 1989). However, during synthesis in a mesophilic organism, the thermophilic proteins may adopt a folding pattern different from the physiological one, as shown by the case of *T. maritima* EF-Tu expressed in *E. coli* (Tiboni *et al.*, 1989). On the other hand, many thermophilic proteins are able to complement defective *E. coli* mutants, implying that they are correctly folded at the host growth temperature (Branlant *et al.*, 1989; Cubellis *et al.* 1990).

Here we have shown that an *E. coli* strain auxotrophic for glutamine is able to grow, albeit slowly, on minimal medium following transformation with a plasmid carrying the *glnA* gene from the extreme thermophilic bacterium *T. maritima*. This is in accordance with *in vitro* evidence that the recombinant subunit proteins are

correctly folded and assembled into a dodecamer during synthesis in *E. coli*. The activation energy calculated from the Arrhenius plot of GS activity evaluated over a wide temperature spectrum is consistent with the *T. maritima* GS being a rather rigid molecule, and this rigidity adversely affecting catalytic rates at temperatures below the physiological optimum for *T. maritima* growth. At high temperature, however, the *T. maritima* enzyme could become sufficiently flexible to be fully active, while retaining enough rigidity to withstand heat denaturation. This would explain the low efficiency of the *in vivo* complementation in the mesophilic host growth conditions.

Although the GSs from different micro-organisms exhibit structural similarities, their activities are regulated by different mechanisms, such as divalent-cation-induced conformational changes, feedback inhibition by various end-products, including glutamine, and covalent modification of the enzyme subunits by an adenylation-deadenylation system (Tyler, 1978). In general, the GSs from enteric bacteria and *Streptomyces* (Reitzer & Magasanik, 1987; Streicher & Tyler, 1981; Fisher & Wray, 1989) are subject to adenylation unlike other Gram-positive bacteria (Deuel & Prusiner, 1974; Janssen *et al.*, 1988), cyanobacteria (Fisher *et al.*, 1981), and the archaeote *Methanobacterium ivanovi* (Bhatnagar *et al.*, 1986). The insensitivity of the *T. maritima* GS transferase activity to SVP treatment indicates that it is not regulated by an adenylation-deadenylation mechanism, a conclusion also supported by the low degree of sequence conservation (4 out of 18 amino acids) around the tyrosine residue which functions as the adenylation site in *E. coli*.

An intriguing aspect of the results is the evidence that the sequence similarity between the GS of *T. maritima* (a bacterium) and the GS of *P. woesei* (an archaeote) is much greater than that between *T. maritima* GS and *E. coli* GS (two members of the same domain). The significance of such obvious discrepancy between the similarities in GS sequences and the phylogenetic placement of the organisms inferred from analysis of 16S rRNA sequence (Woese, 1987) is a matter of speculation.

As far as thermophily is concerned, a comparison of thermophilic and mesophilic GS sequences does not reveal any obvious trend towards replacement of small amino acids with bulkier and more hydrophobic ones in the thermophilic protein, as appears to be the case for GAPDHs from thermophilic and mesophilic sources (Fabry *et al.*, 1989; Shultes *et al.*, 1990).

It has been suggested that in thermophilic proteins, cysteine residues (susceptible to oxidation at elevated temperatures) are replaced in non-essential positions (Harris & Perham, 1968). When the thermophilic GSs were analysed for cysteine content, the number of such

residues was found to be lower (2, 1, 1 respectively for *Thermotoga*, *Pyrococcus* and *Sulfolobus*) than that of *E. coli* (4 residues) and *B. subtilis* (3 residues) GSs. The significance of this finding, however, is questionable if one considers that the GS of *Streptomyces coelicolor* (a mesophile) contains a single cysteine residue (Wray & Fisher, 1988). The cysteine replacement hypothesis is also contradicted by the evidence that the number of cysteine residues in the *T. maritima* GAPDH (Schultes *et al.*, 1990) does not differ significantly from that found in corresponding mesophilic enzyme. This suggests that no uniform strategy can be envisaged to explain protein adaptation to high temperatures.

The only possible signature distinguishing the GSs of the three extreme thermophiles from those of mesophiles is the presence of a tyrosine residue in place of a valine residue within the catalytically important region V of the enzyme. The relevance of this valine to tyrosine change to thermal stability will only be elucidated by site-directed mutagenesis experiments.

This work was supported by grants from the Ministry of Education and by a CNR grant Progetto Finalizzato Biotecnologie e Biostrumentazione, sottoprogetto Bioingegneria Molecolare e Cellulare.

References

- 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.
- BEHRMANN, I., HILLEMANN, D., PUHLER, A., STRAUCH, E. & WOHLLEBEN, W. (1990). Overexpression of a *Streptomyces viridochromogenes* gene (*glnII*) encoding a glutamine synthetase similar to those of eukaryotes confers resistance against the antibiotic phosphinothricyl-alanyl-alanine. *Journal of Bacteriology* **172**, 5326-5334.
- 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.
- BLIN, N. & STAFFORD, D. W. (1976). A general method for the isolation of high molecular weight DNA from eukaryotes. *Nucleic Acids Research* **3**, 2303-2308.
- BOLIVAR, F., RODRIGUEZ, R. L., GREENE, P. J., BETLACH, M. C., HEYNEKER, H. L., BOYER, H. W., CROSA, J. H. & FALKOW, S. (1977). Construction and characterization of new cloning vehicles II. A multipurpose cloning system. *Gene* **2**, 95-113.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.
- BRANLANT, C., OSTER, T. & BRANLANT, G. (1989). Nucleotide sequence determination of the DNA region coding for *Bacillus stearothermophilus* glyceraldehyde-3-phosphate dehydrogenase and of the flanking DNA regions required for its expression in *Escherichia coli*. *Gene* **75**, 145-155.
- 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.

- CUBELLIS, M. V., ROZZO, C., MONTECUCCHI, P. & ROSSI, M. (1990). Isolation and sequencing of a new β -galactosidase-encoding archaeobacterial gene. *Gene* **94**, 89–94.
- CULLIMORE, J. V. & BENNET, M. J. (1988). The molecular biology and biochemistry of plant glutamine synthetase from root nodules of *Phaseolus vulgaris* L. and other legumes. *Journal of Plant Physiology* **132**, 387–393.
- DAGERT, M. & EHRLICH, S. D. (1979). Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene* **6**, 23–28.
- DEUEL, T. F. & PRUSINER, S. (1974). Regulation of glutamine synthetase from *Bacillus subtilis* by divalent cations, feedback inhibitors, and L-glutamine. *Journal of Biological Chemistry* **249**, 257–264.
- FABRY, S., LANG, J., NIERMANN, T., VINGRON, M. & HENSEL, R. (1989). Nucleotide sequence of the glyceraldehyde-3-phosphate dehydrogenase gene from the mesophilic methanogenic archaeobacteria *Methanobacterium bryantii* and *Methanobacterium formicum*. Comparison with the respective gene structure of the closely related extreme thermophile *Methanothermobacter fervidus*. *European Journal of Biochemistry* **179**, 405–413.
- FISHER, R., TULI, R. & HASELKORN, R. (1981). A cloned cyanobacterial gene for glutamine synthetase functions in *Escherichia coli*, but the enzyme is not adenylylated. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 3393–3397.
- FISHER, S. H. & WRAY, L. V. (1989). Regulation of glutamine synthetase in *Streptomyces coelicolor*. *Journal of Bacteriology* **171**, 2378–2383.
- HAIZER, D. J. & MOSES, V. (1978). The enzymes of proline biosynthesis in *Escherichia coli*. Their molecular weights and the problem of enzyme aggregation. *Biochemical Journal* **173**, 219–228.
- HARRIS, J. I. & PERHAM, R. N. (1968). Glyceraldehyde 3-phosphate dehydrogenase from pig muscle. *Nature, London* **219**, 1025–1028.
- HIGUCHI, R., STANG, H. D., BROWNE, J. K., MARTIN, M. O., HUOT, M., LIPELES, J. & SALSER, W. (1981). Human ribosomal RNA gene spacer sequences are found interspersed elsewhere in the genome. *Gene* **15**, 177–186.
- HILL, R. T., PARKER, J. R., GOODMAN, H. J. K., JONES, D. T. & WOODS, D. R. (1989). Molecular analysis of a novel glutamine synthetase of the anaerobe *Bacteroides fragilis*. *Journal of General Microbiology* **135**, 3271–3279.
- HUBER, R., LANGWORTHY, T. A., KONIG, H., THOMM, M., WOESE, C. R., SLEYTR, U. B. & STETTER, K. O. (1986). *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90 °C. *Archives of Microbiology* **144**, 324–333.
- 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.
- KUMADA, Y., TAKANO, E., NAGAOKA, K. & THOMPSON, C. J. (1990). *Streptomyces hygroscopicus* has two glutamine synthetase genes. *Journal of Bacteriology* **172**, 5343–5351.
- LOVE, D. R. & STREIFF, M. B. (1987). Molecular cloning of a β -glucosidase gene from an extremely thermophilic anaerobe in *E. coli* and *B. subtilis*. *Biotechnology* **5**, 384–387.
- LOVE, D. R., FISHER, R. & BERGQUIST, P. L. (1988). Sequence structure and expression of a cloned β -glucosidase gene from an extreme thermophile. *Molecular General Genetics* **213**, 84–92.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MARCK, C. (1988). 'DNA Strider': a 'C' program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. *Nucleic Acids Research* **16**, 1829–1836.
- NAKANO, Y., TANAKA, E., KATO, C., KIMURA, K. & HORIKOSHI, K. (1989). The complete nucleotide sequence of the glutamine synthetase gene (*glnA*) of *Bacillus subtilis*. *FEMS Microbiology Letters* **57**, 81–86.
- PLEDGER, R. J. & BAROSS, J. A. (1991). Preliminary description and nutritional characterization of a chemoorganotrophic archaeobacterium growing at temperatures of up to 110 °C isolated from a submarine hydrothermal vent environment. *Journal of General Microbiology* **137**, 203–211.
- POSSOT, O., SIBOLD, L. & AUBERT, J.-P. (1989). Nucleotide sequence and expression of the glutamine synthetase structural gene, *glnA*, of the archaeobacterium *Methanococcus voltae*. *Research in Microbiology* **140**, 355–371.
- 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.
- REITZER, L. J. & MAGASANIK, B. (1987). Ammonia assimilation and the biosynthesis of glutamine, glutamate, aspartate, asparagine, L-alanine and D-alanine. In *Escherichia coli and Salmonella typhimurium*, pp. 302–320. Edited by F. C. Neidhart. Washington, DC: American Society for Microbiology.
- RICCARDI, G., DE ROSSI, E., DELLA VALLE, G. & CIFERRI, O. (1985). Cloning of the glutamine synthetase gene from *Spirulina platensis*. *Plant Molecular Biology* **4**, 133–136.
- SANANGELANTONI, A. M., BARBARINI, D., DI PASQUALE, G., CAMMARANO, P. & TIBONI, O. (1990). Cloning and nucleotide sequence of an archaeobacterial glutamine synthetase gene: phylogenetic implications. *Molecular General Genetics* **221**, 187–194.
- 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.
- SCHULTES, V., DEUTZMANN, R. & JAENICKE, R. (1990). Complete amino-acid sequence of glyceraldehyde-3-phosphate dehydrogenase from the hyperthermophilic eubacterium *Thermotoga maritima*. *European Journal of Biochemistry* **192**, 25–31.
- SHAPIRO, B. M. & STADTMAN, E. R. (1968). 5'-Adenylyl-O-tyrosine. The novel phosphodiester residue of adenylylated glutamine synthetase from *Escherichia coli*. *Journal of Biological Chemistry* **243**, 3769–3771.
- SHAPIRO, B. M., KINGDOM, H. S. & STADTMAN, E. R. (1967). Regulation of glutamine synthetase. VII. Adenylyl glutamine synthetase: a new form of the enzyme with altered regulatory and kinetic properties. *Proceedings of the National Academy of Sciences of the United States of America* **58**, 642–649.
- SHATTERS, R. G. & KAHN, M. L. (1989). Glutamine synthetase II in *Rhizobium*: reexamination of the proposed horizontal transfer of DNA from eukaryotes to prokaryotes. *Journal of Molecular Evolution* **29**, 422–428.
- SHINE, C. & DALGARNO, L. (1976). Determinant of cistron specificity in bacterial ribosomes. *Nature, London* **254**, 34–38.
- SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503–517.
- STADTMAN, E. R., SMYRNIOTIS, P. Z., DAVIS, J. N. & WITTEMBERG, M. E. (1979). Enzymic procedures for determining the average state of adenylylation of *Escherichia coli* glutamine synthetase. *Analytical Biochemistry* **95**, 275–285.
- STREICHER, S. L. & TYLER, B. (1980). Purification of glutamine synthetase from a variety of bacteria. *Journal of Bacteriology* **142**, 69–78.
- STREICHER, S. L. & TYLER, B. (1981). Regulation of glutamine synthetase activity by adenylylation in the Gram-positive bacterium *Streptomyces cattleya*. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 229–233.
- TIBONI, O., SANANGELANTONI, A. M., CAMMARANO, P., CIMINO, L., DI PASQUALE, G. & SORA, S. (1989). Expression in *Escherichia coli* of the *tuf* gene from the extremely thermophilic eubacterium *Thermotoga maritima*: Purification of the *Thermotoga* elongation factor Tu by thermal denaturation of the mesophile host cell proteins. *Systematic and Applied Microbiology* **12**, 127–133.
- TOUKDARIAN, A., SAUNDERS, G., SELMAN-SOSA, G., SANTERO, E., WOODLEY, P. & KENNEDY, C. (1990). Molecular analysis of the *Azotobacter vinelandii* *glnA* gene encoding glutamine synthetase. *Journal of Bacteriology* **172**, 6529–6539.
- TYLER, B. (1978). Regulation of the assimilation of nitrogen compounds. *Annual Review of Biochemistry* **47**, 1127–1162.
- VIEIRA, J. & MESSING, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259–268.

- WILBUR, W. J. & LIPMAN, D. J. (1983). Rapid similarity searches of nucleic acid and protein data banks. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 726–730.
- WOESE, C. R. (1987). Bacterial evolution. *Microbiological Reviews* **51**, 221–271.
- WRAY, L. V. & FISHER, S. H. (1988). Cloning and nucleotide sequence of the *Streptomyces coelicolor* gene encoding glutamine synthetase. *Gene* **71**, 247–256.
- ZILLIG, W., HOLZ, I., JANEKOVIC, D., KLENK, H.-D., IMSEL, E., TRENT, J., WUNDERL, S., FORJAZ, V. H., COUTINHO, R. & FERREIRA, T. (1990). *Hyperthermus butylicus*, a hyperthermophilic sulfur-reducing archaeobacterium that ferments peptides. *Journal of Bacteriology* **172**, 3959–3965.