Primary structure, partial purification and regulation of key enzymes of the acetyl cycle of arginine biosynthesis in *Bacillus stearothermophilus*: dual function of ornithine acetyltransferase.

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A 3.4 kb *EcoRI* fragment, cloned in *E. coli*, that carries part of a cluster of genes encoding arginine biosynthetic functions of the thermophilic bacterium *Bacillus stearothermophilus*, was sequenced on both strands. The sequence consists of a truncated *argC* gene, an *argJ* region encoding a polypeptide with both *N*-acetylglutamate synthase and ornithine acetyltransferase activities, the *argB* gene and the *N*-terminal part of *argD*. The *argB* gene encodes a 258-amino-acid polypeptide with a deduced *M_r* of 26918. A very high and thermostable *N*-acetylglutamate 5-phosphotransferase activity was detected in extracts of *E. coli argB* mutants transformed with the 3.4 kb fragment on a plasmid. A polypeptide band of *M_r* 27000 was detected by SDS-PAGE of heat-treated extract from such a strain. Both *N*-acetylglutamate synthase and ornithine acetyltransferase are encoded by the same 1290 bp open reading frame. The deduced sequence of 410 amino acids corresponds to a peptide of *M_r* 43349. The subcloned *B. stearothermophilus argJ* can complement a double *argA argE E. coli* mutant to prototrophy. Gel-filtration of a heat-treated extract of the complemented double mutant *E. coli* host showed that *N*-acetylglutamate synthase and ornithine acetyltransferase activities co-elute in a single peak corresponding to *M_r* 110000. Both activities were also heat-inactivated at the same temperature and strongly inhibited by ornithine. These results suggest that both activities can be ascribed to a single protein.

Introduction

Two pathways are known for the *de novo* synthesis of ornithine, a precursor of arginine. In the so-called 'linear' pathway, *N*²-acetyl-L-ornithine is converted into ornithine and acetate by *N*²-acetyl-L-ornithine aminohydrolase (acetylornithinase; EC 3.5.1.16; genetic symbol *argE*). This route is characteristic of *Enterobacteriaceae* among eubacteria, and of *Sulfolobus solfataricus* among archaeobacteria (see Van de Castele *et al.*, 1990). In the so-called 'cyclic' pathway, however,

the acetyl group of *N*²-acetyl-L-ornithine is recycled through acetylation of glutamate to produce *N*²-acetyl-L-glutamate, an earlier precursor of ornithine; this transacetylation is catalysed by ornithine acetyltransferase (*N*²-acetyl-L-ornithine:L-glutamate *N*-acetyl-transferase; EC 2.3.1.35; genetic symbol *argJ*). The cyclic route is used by all other prokaryotes investigated up to now, including the genus *Bacillus* (Sakanyan *et al.*, 1992) and eukaryotic microbes (see Cunin *et al.*, 1986; Davis *et al.*, 1986). *N*-Acetylglutamate synthase (acetyl-CoA:L-glutamate *N*-acetyltransferase; EC 2.3.1.1; genetic symbol *argA*) is common to both pathways but, in the cyclic one, plays a purely anaplerotic role.

A pathway-specific pattern of control of enzyme activity was originally reported by Udaka (1966): in *Enterobacteriaceae*, where the linear pathway operates, acetylglutamate synthase is the first committed step of the biosynthesis and is actually the target of feedback inhibition by arginine (Vyas & Maas, 1963; Cunin *et al.*,

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1986). In organisms using the cyclic pathway, however, the key enzyme inhibited by arginine is either *N*-acetylglutamate 5-phosphotransferase [ATP:*N*-acetyl-L-glutamate 5-phosphotransferase; EC 2.7.2.8; genetic symbol *argB*; Udaka (1966); see Cunin *et al.* (1986) and Davis (1986)] or ornithine acetyltransferase itself (Van de Castele *et al.*, 1990); in that case, acetylglutamate synthase may (as in *Pseudomonas*; Haas *et al.*, 1972) or may not (as in *Thermus*; Van de Castele *et al.*, 1990) be feedback inhibited by arginine. Two extreme thermophiles (*Sulfolobus solfataricus* and *Thermotoga maritima*), respectively using the linear and cyclic pathways, appear to be devoid of feedback inhibition (*ibid.*).

Some considerable time ago, the ability of the *argJ* enzyme to transfer acetyl groups brought about the suspicion that in the organisms using the cyclic pathway, the *de novo* synthesis of acetylglutamate and the transacetylation reaction may not be the exclusive attributes of two different enzymes: a brief report (Chou & Gunsalus, 1971) even stated that in *Pseudomonas putida*, the enzyme specific for the synthesis of acetylglutamate was dispensable. To our knowledge, this statement has not been substantiated by further evidence but, as we shall see, it may have been premonitory of the situation existing in several organisms. Two contrasting reports appeared later on: one by Morris & Thompson (1975), who found in *Chlorella* that the two activities could not be separated during partial purification, and another one by Haas *et al.* (1977), who reported two important observations concerning *P. aeruginosa*. In this organism, the two activities are separable by gel-filtration, and several *argA* mutants could be isolated which were devoid of acetylglutamate synthase activity, displayed normal acetyltransferase activity and had an arginine-less phenotype. Acetylglutamate synthase has been studied to some extent in *P. aeruginosa* (Haas *et al.*, 1972; Haas & Leisinger, 1974) and in *Saccharomyces cerevisiae* (Wipf & Leisinger, 1979) and found to be devoid of acetylornithine:glutamate transacetylation activity (Haas *et al.*, 1972). The transacetylation reaction itself has not been studied extensively in any organism. Mutants defective in *argJ* but not in *argA* have been reported in yeast (see Davis, 1986) and in *Neisseria gonorrhoeae* (Picard & Dillon, 1989). Arginine auxotrophs able to grow on acetylglutamate were reported in yeast (M. Grenson, quoted in Wiame & Dubois, 1976) but not in *Neisseria*. Clearly, a much less ambiguous gene-enzyme relationship had to be established before making conclusions as to the substrate specificity of the *argJ* enzyme and the respective roles played by *argA* and *argJ* in different organisms.

Critical in this respect were a number of recent reports that the same short fragment of DNA from *Bacillus stearothermophilus* (Sakanyan *et al.*, 1987, 1990) or from *N. gonorrhoeae* (Picard & Dillon, 1989) could comple-

ment both *argA* and *argE* (acetylornithinase) *E. coli* auxotrophs, suggesting that, in these organisms at least, a single enzyme can synthesize acetylglutamate from glutamate and either acetyl-CoA or acetylornithine as acetyl donor. In keeping with this suggestion, the shortest *B. stearothermophilus* piece of DNA able to complement *argA* and *argE*, or *argA argE E. coli* mutants was found to be 1350 nt (nucleotides) long (Sakanyan *et al.*, 1992). Most recently, Martin & Mulks (1992) cloned from *Neisseria* and sequenced a single gene (*argJ*) encoding an open reading frame (ORF) of 1218 nt which complements both *E. coli argA* and *argE* auxotrophs.

In the present communication, we present the characterization of the *argJ* gene of *B. stearothermophilus* and report on the regulatory properties and partial purification of the transacetylase. We also present corresponding data concerning the adjacent *argB* gene and the cognate acetylglutamate 5-phosphotransferase, the other key enzyme of the acetyl cycle of arginine biosynthesis. We discuss the metabolic significance of these data.

Methods

Bacterial strains and plasmids. These are listed in Table 1.

Media and growth conditions. *E. coli* strains were grown at 37 °C in L-broth medium and in synthetic M9 (Maniatis *et al.*, 1982) or 132 medium (Falmagne *et al.*, 1965). Concentrations of antibiotics (in µg ml⁻¹) were: ampicillin (Ap), 100, and tetracycline hydrochloride (Tc), 30.

DNA isolation, restriction and ligation. Plasmid DNA was isolated by the cleared lysate method according to Birnboim & Doly (1979). Single-stranded template DNA for sequencing purposes was isolated by polyethylene glycol precipitation and phenol extraction from the supernatant of JM101 cultures infected with M13 derivatives. Restriction enzymes, T4 DNA ligase and M13 DNA were purchased from Boehringer and used according to the manufacturer's recommendations.

Transformation of bacteria. *E. coli* cells were made competent for transformation by CaCl₂ treatment (Dagert & Ehrlich, 1979).

Sequencing strategy. The entire 3.4 kb *EcoRI* fragment of plasmid pAVK1 was cloned into M13mp19 in one orientation. After the creation of an appropriate set of increasing unidirectional deletions according to the method of Dale *et al.* (1985), the sequence of the coding strand of the *arg* cluster of genes was established by the dideoxy chain termination method (Sanger, 1981). The 0.5 and 2.9 kb *EcoRI*-*PstI* subfragments were respectively subcloned into M13mp19 and M13mp18 to sequence the complementary strand using a series of synthetic 20-mer oligonucleotides hybridizing to sites separated by 200 bp. Double-stranded pAVK7 DNA was also used as template to sequence the *argJ*-*argB* region. Ambiguities in sequence reading due to band compression were solved by substituting the analogue C³dGTP for dGTP in the sequencing mixes (Pharmacia sequencing kit).

Enzyme assays. Cells from exponential phase cultures (about 6 × 10⁸ cells ml⁻¹) were harvested by centrifugation (10 min, 7000 g) and washed in 0.9% NaCl. Cells were suspended in 20 mM-potassium phosphate buffer (pH 7.5), disrupted by sonication for 10 min, and the resulting extracts were centrifuged (15 min, 20000 g). For arginine and ornithine inhibition experiments, the crude extracts were passed through Sephadex G-25 columns equilibrated with extraction buffer. All of

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Relevant genotype or phenotype	Source or reference
<i>E. coli</i> K12		
JM101	<i>supE thi-1 Δ(lac-proAB)</i> [F' <i>traD36 proAB lacI^qΔM15</i>]	Yanisch-Perron <i>et al.</i> (1985)
XB25	F ⁻ <i>argB nalA λ⁻ λ^s hsdR</i>	S. Baumberg, University of Leeds, UK
XA4	F ⁻ <i>argA nalA λ⁻ λ^s hsdR</i>	
XA4 <i>argE</i>	as XA4, but <i>argE86::Tn10</i>	
<i>B. stearothermophilus</i>	Prototroph	All Union Collection of Microorganisms, Moscow, Russia
NCIB8224		
Plasmid		
pAVK1	Ap ^r Tc ^r <i>arg' CJBD'</i>	Sakanyan <i>et al.</i> (1992)
pAVK7	Ap ^r <i>arg' CJB'</i>	
M13mp18		Messing (1983)
M13mp19		

these steps were performed at 0–5 °C. Enzymes were assayed at 55 °C. Inhibition studies were performed without pre-incubation.

N-Acetylglutamate synthase and ornithine acetyltransferase were assayed as described in Van de Castele *et al.* (1990), except that 15% (v/v) glycerol, 10 mM-MgCl₂ and 7 mM-aminooxyacetic acid were added to the incubation mixture. Aminooxyacetic acid was added in order to reduce the transamination of glutamate in crude extracts.

N-Acetylglutamate 5-phosphotransferase was measured by the ferric chloride method according to Van de Castele *et al.* (1990).

One enzyme unit is defined as the amount of enzyme that converts 1 μmol substrate to product h⁻¹. Protein concentrations were determined by the Lowry method.

M_r determinations. *M_r* values of native enzymes were determined by gel-filtration on a Sephadex G-200 (2.5 × 30 cm) column (eluted with 20 mM-potassium phosphate buffer, pH 7.5, at a flow rate of 6 ml h⁻¹). The gel-filtration column was calibrated using the following standards: catalase (*M_r* 232 000), aldolase (158 000), albumin (67 000), ovalbumin (43 000), chymotrypsinogen A (25 000) and ribonuclease A (13 700). The elution volume (*V_e*) of each standard was determined from the absorbance at 280 nm. *N*-Acetylglutamate synthase, ornithine acetyltransferase and *N*-acetylglutamate 5-phosphotransferase were detected by activity measurements. The void volume (*V₀*) was determined by blue dextran exclusion.

SDS-PAGE. This was done on 8–25% gradient gels in the Phast System (Pharmacia). Protein bands were visualized by staining with Coomassie brilliant blue.

Results

Sequence of *B. stearothermophilus arg* genes

The cloning of a 3.4 kb *Eco*RI fragment of *B. stearothermophilus* complementing *argA*, *argB* and *argE* auxotrophs of *E. coli* has been reported previously (Sakanyan *et al.*, 1990). This work and a later analysis (Sakanyan *et al.*, 1992) showed that the cloned fragment (in plasmid pAVK1) expressed activities of acetylglutamate synthase (*argA* complementation), acetylglutamate phosphotransferase (*argB* complementation) and ornithine acetyltransferase (*argE* complementation). The entire fragment has now been sequenced (Fig. 1).

Starting from the left-end border (see Fig. 2) the fragment was shown in this work to consist of (i) a sequence homologous to the carboxy-terminal part of the *E. coli*, *B. subtilis* and yeast *argC* gene [encoding *N*-acetyl-L-glutamate 5-semialdehyde:NADP⁺ oxidoreductase (phosphorylating); EC 1.2.1.38; see Parsot *et al.* (1988), Smith *et al.* (1990), Boonchird *et al.* (1991)]; (ii) an open reading frame of 1230 nt, homologous to the recently sequenced *argJ* gene of *Neisseria* [39% identity; see Fig. 3 and Martin & Mulks (1992)]; (iii) a complete *argB* gene homologous to the cognate *E. coli* and yeast genes [41% identity with *E. coli*; Parsot *et al.* (1988), Boonchird *et al.* (1991)]; (iv) an incomplete *argD* gene (encoding *N*²-acetyl-L-ornithine:2-oxoglutarate aminotransferase; EC 2.6.1.11), showing 39% identity with its *E. coli* and yeast homologues (Heimberg *et al.*, 1990). In the shorter pAVK7 plasmid (Sakanyan *et al.*, 1990, 1992), the *argB* gene is incomplete.

Analysis of the sequence

The data are compatible with the notion that the four genes are expressed as part of an operon, though substantiation of this hypothesis awaits sequencing of at least the promoter proximal part and analysis of gene transcription in the native context; these investigations are in progress and their results will be reported elsewhere. Salient features (see Fig. 1) are (i) the presence of putative ribosome binding sites proximal to *argJ*, *argB* and *argD*; (ii) an overlap between the *argJ* stop codon and the *argB* Shine–Dalgarno site and an overlap between the *argB* stop codon and the translational start of *argD*, suggestive of translational coupling; (iii) a codon usage (data not shown) which shows a 10% increase in the usage of G or C in the third position relative to *E. coli*.

The available (for *argC*) sequence data indicate a considerably higher percentage of identity with *B. subtilis* (59.3%) than with *E. coli* (39.7%) and *S. cerevisiae*

<i>argC</i>	10	30	50		<i>argJ</i>	910	930	950
GTGATGTCGGTGTTCATATAAATTGAAATCGAGCGCTTGCCAACTATGACGCGTC					GATGACGATCACAAAACAAACGGGCAAGTGACGGCGTCCCGATGGAACACAGTGTAC			
V D G A V L H K L E I E A L A K Y D A V					M T I T K Q T G Q V T A V A D G T V V T			
	70	90	110			970	990	1010
TTTTTCCGACGCCCGGGGTGTCGGGGAGTGGGCGCGCGCTCGTGTGATCGGGGA					GCCGGAAGGATTTCAAGCGCGCGGGTGAATGCCGGCTGCGGTATTCGAAAAACGATTT			
F F A T P P G V S G E W A P A L V D R G					P E G F Q A A G V N A G L R Y S K N D L			
	130	150	170			1030	1050	1070
GTGAAATGATCGATTATCGGCGATTTCGGTTGAAAGACGCGCGGTACGCACAA					AGGGTATTCTATGCGACGTGCCGCTTCGGCGGCGCGGTGTATACGCAAGCCATTT			
V K V I D L S G D F R L K D G A V Y A Q					G V I L C D V P A S A A A V Y T Q S H F			
	190	210	230			1090	1110	1130
TGGTATGGCGCGAGCGCGCATCTGCGTATTATAGCGGGGGGTGTACGGATTGACC					TCAGCGCGCGCGCTCAAAGTACGCGAGCGAGCCTCGCTGTAGAACAAAATTCACGC			
W Y G R E A A P S A Y L E R A V Y G L T					Q A A P L K V T Q A S L A V E Q K L Q A			
	250	270	290			1150	1170	1190
GAGTGAACCGCGAGCGGTGCGGAGCGCTGCTCTATCCAATCGGGTCTATCCG					GGTCATCGTCAACAGCGCGTGCAGAACCCCTGCAACCGTGCAGAAAGGCTCAAGGAGC			
E W N R E A V R G A V L L S N P G C Y P					V I V N R P C A N A C T G A Q G L K D A			
	310	330	350			1210	1230	1250
ACAGCGACGCTGTGGCTTGGCGCGCTTGTCAAAGAGGATTAAATTAAGGAAGACTCG					TTATGAATGCGTGAATTGTCGCGAAACAGTTTGGCCTGGCGTGCACCATGTGGCCGT			
T A T L L G L A P L V K E G L I K E D S					Y E M R E L C A K Q F G L A L H V A V			
	370	390	410			1270	1290	1310
ATCATGTGACGCGCAATCGGCGTATCGGCGCGGACGGAAGCGCGCTCGGACA					CGCTTCAACGGCGCTAATCGGGAATATTTCGCGATGGAATAATTCGCGCGGCATCAA			
I I V D A K S G V S G A G R K A G L G T					A S T G V I G E Y L P M E K I R A G I K			
	430	450	470			1330	1350	1370
CATTTTCCGAAGTGAACGAAACGTGAAATTTATAAGTGAACGCCCAACAGACATT					ACAGCTTGTCCAGGGTGACGATGGCGATGCGGAGGCGTTTCAACGGCGGATTTTAAAC			
H F S E V N E N V K I Y K V N A H Q H I					Q L V P G V T M A D A E A F Q T A I L T			
	490	510	530			1390	1410	1430
CCGAAATTGAACAAGCGCTGCAGACGTGGAATGAAGCGGTGGCGCGATCACGTTACG					GACCGATACCGTGTGAAGCGCGCTTGTACAAACAAACGATCAGCGGAAACAGTCAAC			
P E I E Q A L Q T W N E A V A P I T F S					T D T V M K R A C Y Q T T I D G K T V T			
	550	570	590			1450	1470	1490
ACTCATTTGATTCGATGACAAGGGCATTTATGGCGACGATTTATCGAAGCGGAAACAA					CGTCGCGGAGCGGAAGGTGCGGGATGATCCATCCGAACATGGCGACGATGCTCGC			
T H L I P M T R G I M A T I Y A K A K Q					V G G A A K G S G M I H P N M A T M L A			
	610	630	650			1510	1530	1550
TCGATTCCGCAACGACTTGTAGATTGTATAAACAAGTTATGAAGTTTCGCGGTTT					ATTCATCACACGCGATGCAATTTTCGTCGCCGCTGCTGCACGCGCGCTCGGTCGAT			
S I S P N D L V D L Y K T S Y E G S P F					F I T T D A N V S S P V L H A A L R S I			
	670	690	710			1570	1590	1610
GTCCGCATCGTCAACTTGGACAGTTTCAGCGACACAAAGACGTGTACGCTCAATAT					TACGACGTTTCGTTTAAACAAATACGCGCGGATACGTCGACAAATGATATGGT			
V R I R Q L G Q F P A T K D V Y G S N Y					T D V S F N Q I T V D G D T S T G N D M V			
	730	750	770			1630	1650	1670
TGCGACATCGGCTCGCTACGATGACGCGACGGAACGGTGCACGGTTCGTCGCTGATC					CGTCGTGATGCAAGCGGTCTTGTCTGAAATGATGAGTTGACGCGCGGATCATCCGGACTG			
C D I G L A Y D E R T E R V T V V S V I					V V M A S G L A G N D E L T P D H P D W			
	790	810	830			1690	1710	1730
GACAACTTGATGAAGCGTCCCGGACGCGGTGCAAACTTCAACTTGATGATGGT					GGAAACTTTTATGAGCGCTCGGAAACGTCGGAAGATTTCGGGAGCAAAATCCGCAA			
D N L M K G A A G Q A V Q N F N L M M G					E N F Y E A L R K T C E D L A K Q I A K			
	850	870	890			1750	1770	1790
TGGGATGAGCGGAGGCGCTCCGATCCTTGGCGATCTATCCGTGAGGGAAGGGAAC					AGACGCGAGGCGGACGAGCTCATTTGAAGTGCCTGCGCGCGCGGCGGAAACCGGATGA			
W D E A E G L R S L P I Y P *					D G E G A T K L I E V R V R G A K T D E			

Fig. 1. Nucleotide and corresponding amino-acid sequences of the 3.4 kb *EcoRI* fragment of pAVK1. Potential ribosome binding sites preceding the predicted initiation codons of the *argJ*, *argB* and *argD* genes are underlined. A few recognition sites for restriction enzymes are indicated.

Fig. 2. Physical map and organization of the 3.4 kb *EcoRI* fragment of pAVK1 carrying part of the *arg* gene cluster of *B. stearothermophilus*. The *argC* (coding for *N*-acetylglutamate 5-semialdehyde dehydrogenase) and *argD* (coding for *N*-acetylornithine 5-aminotransferase) genes are incomplete, missing the N-terminal and C-terminal parts, respectively. Gene *argJ* encodes an active ornithine acetyltransferase that also displays *N*-acetylglutamate synthase activity; *argB* encodes an intact *N*-acetylglutamate 5-phosphotransferase.

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Fig. 3. Comparison of the entire amino acid sequences of the *B. stearothermophilus* and *N. gonorrhoeae* ornithine acetyltransferases. The sequences were compared using the FASTA computer program of Pearson & Lipman (1988). Dashes correspond to gaps which have been introduced to align the two sequences. The symbols ':' and '.' indicate identical and similar residues, respectively. B.s., *Bacillus stearothermophilus*; N.g., *Neisseria gonorrhoeae*.

(32.2%). The truncated *argC* sequence contains the conserved cysteine (nt 292 to 294) already reported by Parsot *et al.* (1988) in *E. coli*, *B. subtilis* and *S. cerevisiae*.

Using the FASTA program (Pearson & Lipman, 1988), we could detect no significant similarity between *argJ* and *E. coli argA* (previously sequenced by Brown *et al.*,

Fig. 4

Fig. 5

Fig. 4. Thermostability of *N*-acetylglutamate synthase and ornithine acetyltransferase activity. Dialysed extract (50 mg protein ml⁻¹ in 50 mM-potassium phosphate buffer, pH 7.5) of strain XA4 *argE* (pAVK7) was incubated for 20 min at various temperatures. The residual *N*-acetylglutamate synthase (○) and ornithine acetyltransferase (●) activities were measured under the standard assay conditions. The enzyme activity of the extract without pre-incubation or after 20 min pre-incubation at 25 °C was 24 units ml⁻¹ for *N*-acetylglutamate synthase and 68 units ml⁻¹ for ornithine acetyltransferase.

Fig. 5. Sephadex G200 gel-filtration of *N*-acetylglutamate synthase and ornithine acetyltransferase activity. Crude extract (50 mg protein ml⁻¹ in 50 mM-potassium phosphate buffer, pH 7.5; total vol. 25 ml) of strain XA4 *argE* (pAVK7) was incubated for 20 min at 63 °C. Precipitated protein was eliminated by centrifugation and the supernatant was concentrated by ultrafiltration. The heat-treated extract (about 100 mg protein in 1.5 ml) was applied onto the Sephadex G-200 column and eluted with 50 mM-potassium phosphate buffer, pH 7.5. *N*-Acetylglutamate synthase (○) and ornithine acetyltransferase (●) activities were measured in the elution fractions under the standard assay, conditions. —, Absorbance at 280 nm.

1987), as already noted by Martin & Mulks (1992) in the case of *Neisseria*.

Thermostability and partial purification of ornithine acetyltransferase

E. coli argA argE double mutants complemented by pAVK7 display considerably higher specific activities of ornithine acetyltransferase and *N*-acetylglutamate synthase than *B. stearothermophilus* itself (Sakanyan *et al.*, 1992), which allowed us to investigate the enzyme more easily than in the native background and safe from interference by any additional activity that could be responsible for acetylglutamate synthesis in the mother strain. Actually, there are reasons to think that such an activity exists in extracts of *B. stearothermophilus*: data from Sakanyan *et al.* (1992) show that the synthesis of *N*-acetylglutamate synthase is not repressible in *B. stearothermophilus*, whereas the specific activity of ornithine acetyltransferase decreased fourfold when arginine is added to the growth medium. However, the level of *N*-acetylglutamate synthase measured in *B. stearothermophilus* extracts is low as compared to the activity observed in pAVK1 transformed *E. coli* strains. In this regard, it should be mentioned that such a low level of activity could be due to the presence in *B. stearothermophilus* extracts of an aminoacylase with broad range substrate specificity, and bearing

considerable hydrolysing activity towards *N*-acetyl-L-glutamate, the product of the reaction catalysed by *N*-acetylglutamate synthase (Sakanyan *et al.*, 1992; unpublished work).

The thermal stabilities of *N*-acetylglutamate synthase and ornithine acetyltransferase were tested in extracts of *E. coli* carrying the pAVK7 plasmid. The results are depicted in Fig. 4, where it can be seen that both activities are inactivated by a 20 min incubation within the same short range of temperatures around 70 °C. Whereas the ornithine acetyltransferase activity remains unaffected at temperatures between 20 and 65 °C, the *N*-acetylglutamate synthase activity increased threefold after pre-incubation at temperatures between 55 and 65 °C, as compared to the activity measured without pre-incubation or after 20 min pre-incubation at 25 °C. This deviation in the behaviour of the two activities is unexpected for a bifunctional enzyme. It could, for example, be due to a re-folding of the enzyme upon pre-incubation or, since this experiment was done with crude extract, to the destruction by heat treatment of a factor that interferes with the assay of *N*-acetylglutamate synthase activity. Clearly, the interpretation of this result awaits further analysis of the behaviour of the two activities with highly purified enzyme.

However, when a heat-treated extract of *E. coli* XA4*argE*(pAVK7) was submitted to gel-filtration on Sephadex G200 (Fig. 5.), *N*-acetylglutamate synthase

Table 2. Thermal stability of *B. stearo-thermophilus* *N*-acetylglutamate 5-phosphotransferase synthesized in *E. coli*

Incubation buffer was 50 mM-potassium phosphate, pH 7.5. Protein concentration was about 10 mg ml⁻¹. The half-lives were deduced from the respective semi-log plots.

Protector	Concn (mM)	Half-life at 70 °C (min)
None	—	1.5
MgATP	25	13
<i>N</i> -Acetyl-L-glutamate	50	19
MgATP + <i>N</i> -acetyl-L-glutamate	25 + 50	183

and ornithine acetyltransferase activities were eluted at the same position (corresponding to an M_r of 110000) and their ratio remained almost constant in the fractions that had significant activities (fraction nos 34 to 50). When a similar preparation was applied onto a DEAE-Sephadex column and eluted with a linear gradient of potassium phosphate buffer (pH 7.5; 0.05 to 0.5 M), both activities co-eluted at a concentration of about 0.25 M (not shown).

Feedback inhibition of *N*-acetylglutamate synthase and ornithine acetyltransferase

N-Acetylglutamate synthase and ornithine acetyltransferase activities are but weakly inhibited by arginine, in extracts of both *E. coli* (pAVK7) and *B. stearo-thermophilus*, and the apparent K_m for acetylornithine at 10 mM-glutamate is 0.4 mM (Sakanyan *et al.*, 1992). This weak inhibition is unlikely to be physiologically significant; we found, however, that L-ornithine is a potent inhibitor of both activities. At saturating concentrations of glutamate (20 mM) and of either acetyl-CoA (2 mM) or acetylornithine (10 mM), the apparent K_i values for ornithine were 0.02 mM and 0.2 mM, respectively.

Thermostability and properties of *N*-acetylglutamate 5-phosphotransferase

A very high *N*-acetylglutamate 5-phosphotransferase activity was detected in extracts of an *E. coli* *argB* mutant (strain XB25) harbouring plasmid pAVK1 [210 units (mg protein)⁻¹, as compared to 0.3 unit (mg protein)⁻¹ in *B. stearo-thermophilus* extracts]. The thermostability of *N*-acetylglutamate 5-phosphotransferase activity was increased in the presence of its substrates (Table 2), allowing partial purification, up to sixfold, by a heat treatment (20 min at 70 °C in the presence of 25 mM-MgATP and 50 mM-acetylglutamate). SDS-PAGE of such a heat-treated extract revealed a polypeptide band,

Fig. 6. SDS-PAGE of *B. stearo-thermophilus* *N*-acetylglutamate 5-phosphotransferase synthesized in *E. coli*. Extracts (about 10 mg protein ml⁻¹) of *E. coli* XB25 and XB25(pAVK1) were heat-treated as stated in the text. Precipitated protein was eliminated by centrifugation and samples were heat-treated at 100 °C for 5 min with an equal volume of SDS (5%, w/v) and β -mercaptoethanol (10%, v/v). Lane 1, heat-treated XB25(pAVK1) extract; lane 2, heat-treated XB25 extract; lane 3, standard proteins with the subunit M_r indicated (phosphorylase b (94000), albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100) and α -lactalbumin (14400)).

corresponding to an M_r of 27000, which is absent in heat-treated extract of strain XB25 devoid of plasmid pAVK1 (Fig. 6). The M_r of the enzyme, as estimated by gel-filtration on Sephadex G200, is around 29000 (in 50 mM-potassium phosphate buffer, pH 7.5) and 55000 in the presence of 10 mM-acetylglutamate in the elution buffer. The enzyme was not inhibited by L-ornithine or L-arginine, nor by both amino acids, up to the 50 mM range.

Discussion

In *B. stearo-thermophilus* a single open reading frame (the *argJ* gene), originally identified by its dual complementation pattern of both *argA* and *argE* *E. coli* mutants, was found to encode a transacetylase which catalyses two activities, both of them involved in the cyclic version of arginine biosynthesis: the synthesis of acetylglutamate from glutamate and acetyl-CoA and the production of ornithine by a transacetylation between acetylornithine and glutamate. The ORF is part of a cluster of functionally related genes that we have identified. It is delineated on the promoter proximal end by a sequence homologous to the *argC* gene of other

bacteria and yeast (Parsot *et al.*, 1988; Smith *et al.*, 1990) and on the distal site by the *B. stearothermophilus* *argB* gene; the latter is followed by a sequence homologous to the *E. coli* and yeast genes encoding ornithine aminotransferase (*E. coli* *argD*).

A bifunctional *argJ* gene has also been identified in *N. gonorrhoeae* by a similar dual complementation pattern (Picard & Dillon, 1989; Martin & Mulks, 1992). This gene and the *B. stearothermophilus* cognate sequence share 39% identical residues, a clear indication of homology; therefore, even though *N*-acetylglutamate synthase assays have not been reported for the reaction catalysed by the *argJ* protein in *Neisseria*, there is little doubt that the two organisms use very similar enzymes. Preliminary evidence from this laboratory (M. Baetens, unpublished) indicates that *Thermus aquaticus* and *T. thermophilus* also have a bifunctional *argJ* gene.

Ornithine, and not arginine, appears to be the significant metabolite controlling the enzyme, the *N*-acetylglutamate synthase reaction being by far the most sensitive one: a 0.02 mM apparent K_i with respect to 0.2 mM for the ornithine acetyltransferase reaction. *B. stearothermophilus* has an inducible arginase, converting arginine into ornithine and urea (M. Demarez & C. Legrain, unpublished). It is therefore reasonable to assume that excess arginine leads to the build-up of an ornithine pool able to inhibit *argJ* activity; this remains to be demonstrated, however. On the other hand, acetylglutamate phosphotransferase is not feedback-inhibited in *B. stearothermophilus*, in contrast to many organisms endowed with an acetyl cycle. In terms of metabolic efficiency, this would be understandable inasmuch as the control exerted by ornithine on the bifunctional *argJ* enzyme is efficient enough to curtail the flow of arginine. Considering that the apparent K_i values mentioned above were obtained in the presence of saturating concentrations of substrates, this is not an unreasonable assumption.

At first sight, the bifunctionality of the *argJ* protein would appear to make an *argA* function superfluous even though there are genetic arguments from *Neisseria* (the complementation of *argA* *E. coli* mutants by DNA unlinked to *argJ*; Martin & Mulks, 1992) and enzyme data for *B. stearothermophilus* (Sakanyan *et al.*, 1992) that these organisms possess such a function. However, there is presently no evidence that the *N*-acetylglutamate synthase activity of the transacetylase is actually used *in vivo*; this remains an important issue. Presently, *P. aeruginosa* is the only organism endowed with a cyclic pathway where the *argA* function has been well enough characterized to comment further on this problem: the *argA* enzyme does not use acetylornithine as substrate, *argA* mutants have an arginine-less phenotype, they display no *N*-acetylglutamate synthase activity but

normal ornithine acetyltransferase activity (Haas & Leisinger, 1974; Haas *et al.*, 1977). In *P. aeruginosa*, therefore, one would tend to conclude that *argJ* is not bifunctional. To further substantiate that statement requires characterization of the *argJ* gene and of the cognate protein, however. It may be noted here that in *B. subtilis* a particular subfragment of the cluster of six *arg* genes cloned by Mountain *et al.* (1986) complements *argE* mutants but not *argA* mutants of *E. coli*. Now that it is known that *B. subtilis*, like *B. licheniformis* and *B. stearothermophilus*, uses the cyclic pathway of arginine biosynthesis (Sakanyan *et al.*, 1992), this pattern could be explained if the complementing fragment contained a truncated *argJ* gene having lost the *N*-acetylglutamate synthase function, creating a situation at least superficially reminiscent of the pattern described above for *P. aeruginosa*. Comparing and manipulating cloned *argJ* genes will settle the matter.

It is striking that the *argJ* genes from *Neisseria* and *Bacillus*, despite their *N*-acetylglutamate synthase function, display no recognizable homology with the *argA* gene from *E. coli*. Less surprising is the absence of homology with *argE* which, on the other hand, has been recognized as probably being homologous to the genes for a carboxypeptidase of *Pseudomonas* and for the succinyldiaminopimelate desuccinylase of *E. coli* (Boyen *et al.*, 1992; Meinzel *et al.*, 1992). No sign of homology can be detected either between *argJ* and the *argE* complementing sequence cloned from *Leptospira*, the origin of which remains a mystery (Zuerner & Charon, 1988).

We estimate the M_r of the native transacetylase as 110000 on the basis of gel-filtration. This suggests that the protein is a dimer; indeed, the predicted M_r of the encoded *argJ* polypeptide is 43349, which is in keeping with the size of the subunit estimated by Martin & Mulks (1992) by expressing *Neisseria argJ* in minicells. As estimated by gel-filtration in the presence of *N*-acetylglutamate and MgATP, the *argB* protein has a M_r of 55000, and could therefore be a dimer (the predicted M_r of the encoded *argB* polypeptide is 26918, close to the value found in *E. coli*). The sequence indicates that the *argB* protein is not produced from a precursor common to *argB* and *argC* as in yeast (Boonchird *et al.*, 1991). It will be interesting to see whether this pattern also prevails in more extreme thermophiles, considering the thermolability of acetylglutamate phosphate. At any rate, some form of channeling for this molecule would be expected at higher temperatures.

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