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, 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, 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, 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, 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, N2-acetyl-L-ornithine is converted into ornithine and acetate by N2-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 Casteele et al., 1990). In the so-called ‘cyclic’ pathway, however, the acetyl group of N2-acetyl-L-ornithine is recycled through acetylation of glutamate to produce N2-acetyl-L-glutamate, an earlier precursor of ornithine; this transacetylation is catalysed by ornithine acetyltransferase (N2-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 Casteele et al., 1990); in that case, acetylglutamate synthase may (as in Pseudomonas; Haas et al., 1972) or may not (as in Thermus; Van de Casteele 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 transacylation reaction may not be the exclusive attributes of two different enzymes: a brief report (Chou & Günsalus, 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 gelfiltration, 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 acylornithine-glutamate transacylation activity (Haas et al., 1972). The transacylation 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 Wiamé & 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 was 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 complement both argA and argE (acylornithinase) E. coli auxotrophs, suggesting that, in these organisms at least, a single enzyme can synthesize acetylglutamate from glutamate and either acetyl-CoA or acylornithine 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 transacylase. 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 (Dugert & 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 theideoxy 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 C’dGTP 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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td>E. coli K12</td>
<td>supE thi-1 Δ(lac-proAB)</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>JM101</td>
<td>[F' traD36 proAB lacI*ZAM15]</td>
<td></td>
</tr>
<tr>
<td>XB25</td>
<td>F' argB nalA λ− λ− hsdR</td>
<td>S. Baumberg, University of Leeds, UK</td>
</tr>
<tr>
<td>XA4</td>
<td>F' argA nalA λ− λ− hsdR</td>
<td>Sakanyan et al. (1992)</td>
</tr>
<tr>
<td>XA4 argE</td>
<td>as XA4, but argE86::Tn10</td>
<td></td>
</tr>
<tr>
<td>B. stearothermophilus</td>
<td>Prototroph</td>
<td>All Union Collection of Microorganisms, Moscow, Russia</td>
</tr>
<tr>
<td>NCIB8224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAVK1</td>
<td>Ap' Tc' arg'CJB'</td>
<td>Sakanyan et al. (1992)</td>
</tr>
<tr>
<td>pAVK7</td>
<td>Ap' arg'CJB'</td>
<td></td>
</tr>
<tr>
<td>M13mp18</td>
<td>Ap' arg'CJB'</td>
<td>Messing (1983)</td>
</tr>
<tr>
<td>M13mp19</td>
<td>Ap' arg'CJB'</td>
<td></td>
</tr>
</tbody>
</table>

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 Casteele et al. (1990), except that 15% (v/v) glycerol, 10 mM-MgCl₂ and 7 mM-aminoxyacetic acid were added to the incubation mixture. Aminoxyacetic 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 Casteele 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, determinations. Mₜ 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 (Mₜ 232000), aldolase (158000), albumin (67000), ovalbumin (43000), chymotrypsinogen A (25000) and ribonuclease A (13700). The elution volume (Vₑ) 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 (Vᵥ) 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 EcoRI 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: NAD⁺ 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 N²-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
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.
The truncated \textit{argC} sequence contains the conserved cysteine (nt 292 to 294) already reported by Parsot \textit{et al.} (1988) in \textit{E. coli}, \textit{B. subtilis} and \textit{S. cerevisiae}.

Using the \textit{FASTA} program (Pearson \& Lipman, 1988), we could detect no significant similarity between \textit{argJ} and \textit{E. coli argA} (previously sequenced by Brown \textit{et al.}, 1982).
In this regard, it should be mentioned that such a low level of N-acetylglutamate synthase measured in pAVK1 transformed strain is not repressible in the case of B. stearothermophilus. The synthesis of ornithine acetyltransferase decreased fourfold when ornithine is added to the growth medium. However, the ornithine acetyltransferase and N-acetylglutamate synthase 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.

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 synthase 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 XA4argE(pAVK7) was submitted to gel-filtration on Sephadex G200 (Fig. 5), N-acetylglutamate synthase activity was found to display considerable 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).
Table 2. Thermal stability of *B. stearothermophilus* N-acetyl glutamate 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.

<table>
<thead>
<tr>
<th>Protector</th>
<th>Concen (mm)</th>
<th>Half-life at 70°C (min)</th>
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<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>1.5</td>
</tr>
<tr>
<td>MgATP</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>N-Acetyl-L-glutamate</td>
<td>50</td>
<td>19</td>
</tr>
<tr>
<td>MgATP + N-acetyl-L-glutamate</td>
<td>25 +</td>
<td>183</td>
</tr>
</tbody>
</table>

and ornithine acetyltransferase activities were eluted at the same position (corresponding to an *M*<sub>r</sub> 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-Sepharose 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. stearothermophilus*, and the apparent *K<sub>i</sub>* 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<sub>i</sub>* 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)<sup>−1</sup>, as compared to 0.3 unit (mg protein)<sup>−1</sup> in *B. stearothermophilus* 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, corresponding to an *M*<sub>r</sub> of 27000, which is absent in heat-treated extract of strain XB25 devoid of plasmid pAVK1 (Fig. 6). The *M*<sub>r</sub> 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. stearothermophilus* 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 transacylase 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 transacylation 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. steatorhophilus 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. steatorhophilus 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. thermodilis 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 far the most sensitive one: a 0.02 mM apparent \( K_a \) with respect to 0.2 mM for the ornithine acetyltransferase reaction. B. steatorhophilus 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. steatorhophilus, in contrast to many organisms endowed with an acetyl enzyme. 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 \( K_a \) 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 superfluos 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. steatorhophilus (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 transacytase is actually used \textit{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 acetylyornithine 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. steatorhophilus, 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 carboxypektidase of \textit{Pseudomonas} and for the succinylidiaminopimelate desuccinylase of \textit{E. coli} (Boyen et al., 1992; Meinnel et al., 1992). No sign of homology can be detected either between argJ and the argE complementing sequence cloned from \textit{Leptospira}, the origin of which remains a mystery (Zuerner & Charon, 1988).

We estimate the \( M_r \), of the native transacytase 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 \textit{Neisseria arg}J 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 \textit{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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