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Aspartate carbamoyltransferase from a psychrophilic deep-sea bacterium, *Vibrio* strain 2693: properties of the enzyme, genetic organization and synthesis in *Escherichia coli*

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The aspartate carbamoyltransferase (ATCase) genes of psychrophilic *Vibrio* strain 2693 were cloned by complementation in *Escherichia coli* and the enzyme was partly characterized. The genes constitute a *pyrBI* operon homologous to the cognate structure in *E. coli* where *pyrB* and *pyrI* respectively encode the catalytic and the regulatory chains of ATCase. The strong sequence similarities noted between *Vibrio* and *E. coli* ATCases include extensive conservation of residues involved in interactions between subunits, suggesting that the two enzymes have very similar tertiary and quaternary structures. *Vibrio* ATCase is, however, not activated by ATP and not synergistically inhibited by CTP and UTP. It is also much more thermolabile than *E. coli* ATCase. With respect to *Pyrococcus abyssi* and *E. coli* ATCases, *Vibrio* ATCase presents marked differences in composition which could be related to its psychrophilic character. The results of these structural and functional comparisons indicate that *Vibrio* 2693 ATCase is a suitable model for biochemical studies on structure–function relationships in a 'cold' allosteric enzyme. The operon is expressed from a promoter which is immediately followed by a pyrimidine-rich leader ORF terminating within a putative transcription attenuator. These genetic and enzymic data strengthen the evolutionary relationship already noted between Vibrionaceae and Enterobacteriaceae.

Keywords: aspartate carbamoyltransferase, psychrophiles, *Vibrio*

INTRODUCTION

The study of enzymic catalysis at low temperatures is gathering momentum (for reviews, see Russell, 1990, 1992; Feller *et al.*, 1996; Gerday *et al.*, 1997). One of the reasons for this increased interest in 'cold enzymes' is the large body of knowledge acquired during the last two decades on proteins from thermophilic organisms. Proteins functioning at high temperature are the result of a compromise between the intermolecular forces conferring enough rigidity to ensure stability and the need for functional flexibility (Jaenicke, 1995). On the other hand the architecture of a cold enzyme must ensure enough flexibility to make catalysis possible at a reduced energy cost (Feller *et al.*, 1996). We would expect this need for high flexibility at low temperature to be paralleled by a decrease in stability. To a certain extent therefore, achievement of psychrophily could require molecular strategies converse to those adopted for thermophily. However, the latter appear multiple and are not yet clearly defined; there is thus a need to conduct research specifically focused on the molecular basis for psychrophily. At least two other reasons justify the study of enzymes adapted to cold: (1) the prospect of monitoring stability and catalysis independently (i.e. to produce stable enzymes active at low temperature) is of obvious biotechnological interest; and (2) several key metabolic enzymes are allosteric proteins and are

Abbreviations: ATCase, aspartate carbamoyltransferase; CP, carbamoyl-phosphate.

The EMBL accession number for the sequence reported in this paper is Y09786.

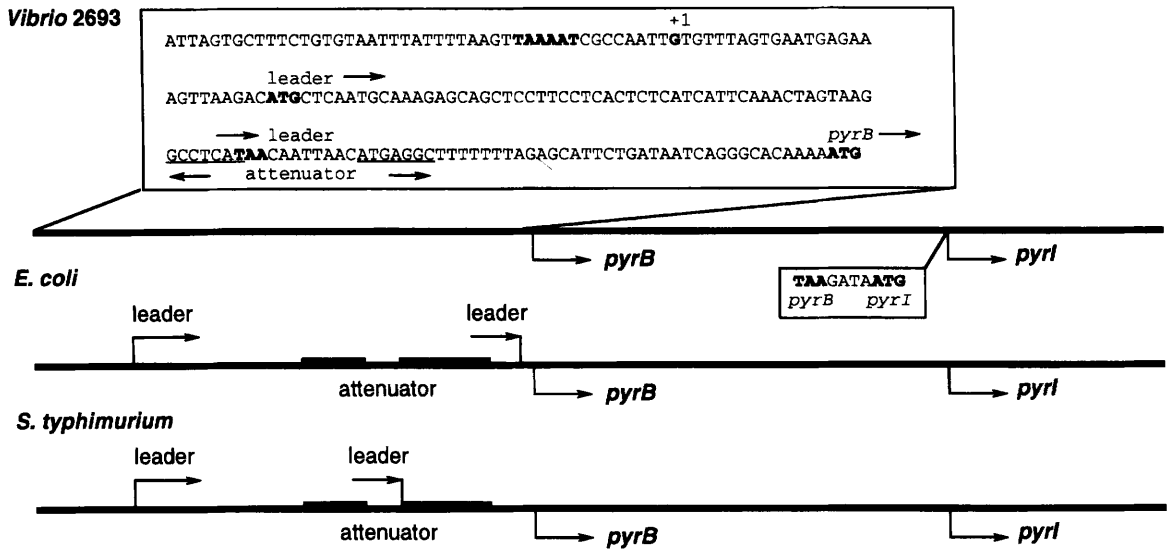

Fig. 1. Comparison of the promoter regions of the *pyrBI* operons in *Vibrio* 2693, *E. coli* and *S. typhimurium*. In the *Vibrio* sequence, bold characters indicate the −10 promoter element (TAAAAT), the experimentally determined transcription start point (+1) and the start and stop codons of the leader ORFs. Inverted repeats are underlined.

therefore subject to the transmission of long-range intramolecular signals – this property emphasizes the need for structural flexibility.

Aspartate carbamoyltransferase (ATCase; EC 2.1.3.2), which catalyses the committed step of pyrimidine biosynthesis, is a particularly suitable enzyme to address the questions outlined above. The structure of *Escherichia coli* ATCase is known at high resolution (2·4 Å; Lipscomb, 1994) and several other ATCases, including thermophilic ones, are currently being investigated (Van de Casteele *et al.*, 1994, 1997a, b; Purcarea *et al.*, 1997; Cunin, 1997). Enterobacterial ATCases belong to the so-called class B ATCases which are composed of two homotrimers of catalytic chains (c3) held together by their association with three homodimers of regulatory chains (r2) harbouring the binding sites for the nucleotide effectors: CTP, an inhibitor, ATP, an activator and UTP which, in synergy with CTP, almost completely inhibits the enzyme (see Wild & Wales, 1990). The structural genes for the c and r subunits have been designated *pyrB* and *pyrI*, respectively; they constitute the *pyrBI* operon (reviewed by Neuhard & Kelln, 1996). Here we report on the properties of the first psychrophilic ATCase to be characterized at the enzymic and genetic level, a prerequisite to structural studies.

METHODS

Bacterial strains and plasmids. The host organism was psychrophilic *Vibrio* strain 2693 isolated by H. Rüger (Alfred-Wegener-Institut für Polar- und Meeresforchung, Bremerhaven, Germany) from the upper sediment layer of the deep Atlantic (−2815 m). The organism is unable to grow above 14 °C and has a maximal growth rate at 6 °C. *E. coli* C600pyrBI$_{1510}$ [Δ(*pro lac argF*) *argI*$^+$ *pyrBI*$_{1510}$ r$^-$ m$^-$] was from this laboratory. *E. coli* strain TG1 [*supE hsd*Δ*5 thi* Δ(*lac–proAB*) F′(*traD36 proAB*$^+$ *lacI*^{q}*lacZ*ΔM15)] was used as host for recombinant plasmids and for single-strand phagemid-DNA rescue. The *E. coli* expression plasmid pTrc99A was from Pharmacia and pBluescript II KS+ was from Stratagene.

Chemicals. Carbamoylphosphate (CP), L-aspartic acid, CTP, UTP, ATP, AMP, UMP, GMP and GTP were from Sigma and oligonucleotides from Gibco-BRL. Restriction enzymes and T4 DNA ligase were from Boehringer Mannheim.

Growth media. Marine Broth 2216 was from Difco. Arginine- and uracil-free (AUF) rich synthetic medium was as described by Piérard & Wiame (1964).

Culture conditions. *E. coli* was grown at 37 °C in 853 broth (Glansdorff, 1965). Ampicillin was used at 50 μg ml^{-1}. *Vibrio* 2693 was grown aerobically at 6 °C in Marine Broth or AUF rich synthetic medium (with artificial sea water as minimal base; Rüger, 1988) to mid-exponential phase (about 5 × 10^8 cells ml^{-1}).

DNA manipulations and transformation. *Sau*3A-digested DNA from *Vibrio* cells grown in Marine Broth was ligated with vector pTrc99A, pre-digested with *Bam*HI, and dephosphorylated by incubating for 4 h at room temperature in the presence of ATP and ligase. Transformation conditions were as described by Dagert & Ehrlich (1978). Small-scale plasmid preparations were obtained by the clear lysate procedure (Birnboim & Doly, 1979). Large-scale preparations were recovered by Nucleobond AXPC100 (Macherey-Nagel).

Sequencing strategy. The *Sac*I–*Pst*I 2·4 kb fragment of plasmid pZYF70 was digested with *Sau*3A and subcloned into the *Bam*HI site of pBluescript II KS+ to generate a series of fragments. Single-strand phage template was isolated and purified according to Messing (1983). Nucleotide sequencing was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977), using the T7 sequencing kit from Pharmacia. Synthetic oligonucleotides were used as primers to sequence the templates prepared from pZYF70.

Southern hybridization. The blotting hybridization technique of Southern (1975) was applied at 68 °C using random primed

probe labelling and hybridization conditions as specified for the DIG labelling and detection system (Boehringer Mannheim).

Primer extension. The antisense oligonucleotide 5′ GATGAGAGTGAGGAAGGAGC 3′ (corresponding to positions +64 to +45 of the *pyrBI* operon) was used for extension by reverse transcriptase. The protocol was as described by Triezenberg (1995). Hybridization was performed at 42 °C.

Enzyme assays. Samples of cell-free extracts obtained by sonic disruption of mid-exponential phase cells were incubated at the required temperature for 10 min in the presence of 20 mM L-aspartate, 5 mM CP and 50 mM Tris/HCl buffer, pH 9·0. The carbamoylaspartate formed was assayed colorimetrically as described by Foltermann *et al.* (1986). The influence of nucleotides on the rate of the reaction was determined as described by Xi *et al.* (1991). The specific ATCase activity was expressed as units (mg protein)$^{-1}$, where a unit was the amount of enzyme which synthesized 1 μmol carbamoylaspartate h^{-1}. Protein concentrations were determined by the Lowry method.

For measurements of ATCase thermostability, cell-free extracts (in 50 mM Tris/HCl buffer, pH 9·0) were incubated in the absence of substrates for 15 min at various temperatures and kept at 0 °C for at least 5 min. Samples were taken and their activity was measured under standard assay conditions at 30 °C.

RESULTS AND DISCUSSION

ATCase activity in *Vibrio* 2693

Vibrio 2693 is prototrophic and grows with a doubling time of 8·5 h at 6 °C (optimal growth temperature) in AUF rich synthetic medium (Liang, 1997). In this medium the specific activity of ATCase was 8–9 units (mg protein)$^{-1}$. The presence of arginine and/or uracil in the medium did not influence this value.

Cloning and sequencing *Vibrio* 2693 ATCase genes

Vibrio 2693 DNA partially digested with enzyme *Sau*3A was used to construct a genomic DNA library in expression vector pTrc99A as described in Methods. A *pyrBI* deletion mutant of *E. coli* was transformed by library DNA and Ura$^+$ colonies were selected for on uracil-free rich synthetic medium at 30 °C. One such clone, found to contain an insert of about 12 kb, was analysed further. After successive fragmentation with enzymes *Sac*I and *Pst*I, a 2·4 kb *Sac*I–*Pst*I insert was isolated that still complemented the *E. coli pyrBI* mutant in either orientation and in the same vector. Plasmid pZF70, harbouring this fragment in the orientation opposite to that of the pTrc promoter carried by the vector, was used for further studies.

Sequencing 1765 bp from this fragment revealed a cluster of two ORFs homologous to the *pyrB* and *pyrI* genes, respectively, of *E. coli* and other organisms (see below and Fig. 3). Southern blotting experiments (data not shown) confirmed that the cloned fragment came from *Vibrio* 2693; under stringent conditions, the fragment did not hybridize with *E. coli pyrBI* DNA.

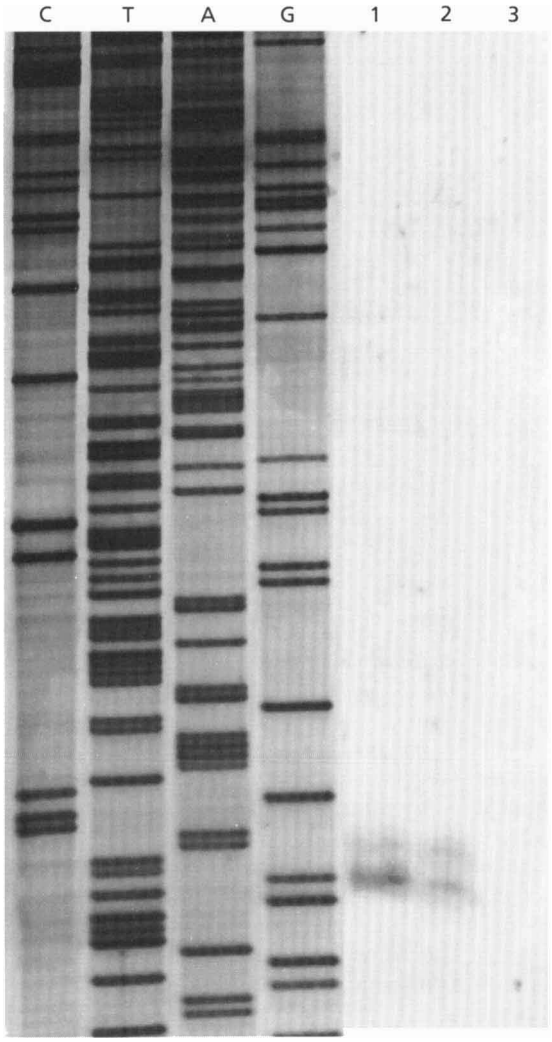

Fig. 2. Analysis of the transcription start sites at the *pyrBI* promoter *in vivo* by primer extension. Lanes: 1 and 2, different RNA preparations; 3, no RNA.

Structure of the control region and start point of transcription

The *pyrB* and *pyrI* homologues found in *Vibrio* are preceded by a control region which extends 141 nt upstream from the putative *pyrB* translation initiation codon (Fig. 1). Indeed, primer extension experiments located a predominant transcription start at a G residue which is itself preceded by a putative −10 TAAAAT sequence element (Fig. 2). No corresponding −35 motif could be identified at a canonical distance, however. Since this is the first promoter to be characterized in this organism, it appears premature to comment on the possible significance of this observation. Between the experimentally determined transcription start point and the putative translation initiation codon, the sequence presents a 21 codon leader ORF preceded at 11 nt by a possible ribosome-binding site (a GAG triplet) and terminated by a TAA codon located within a region presenting the features typical of a *ρ*-independent

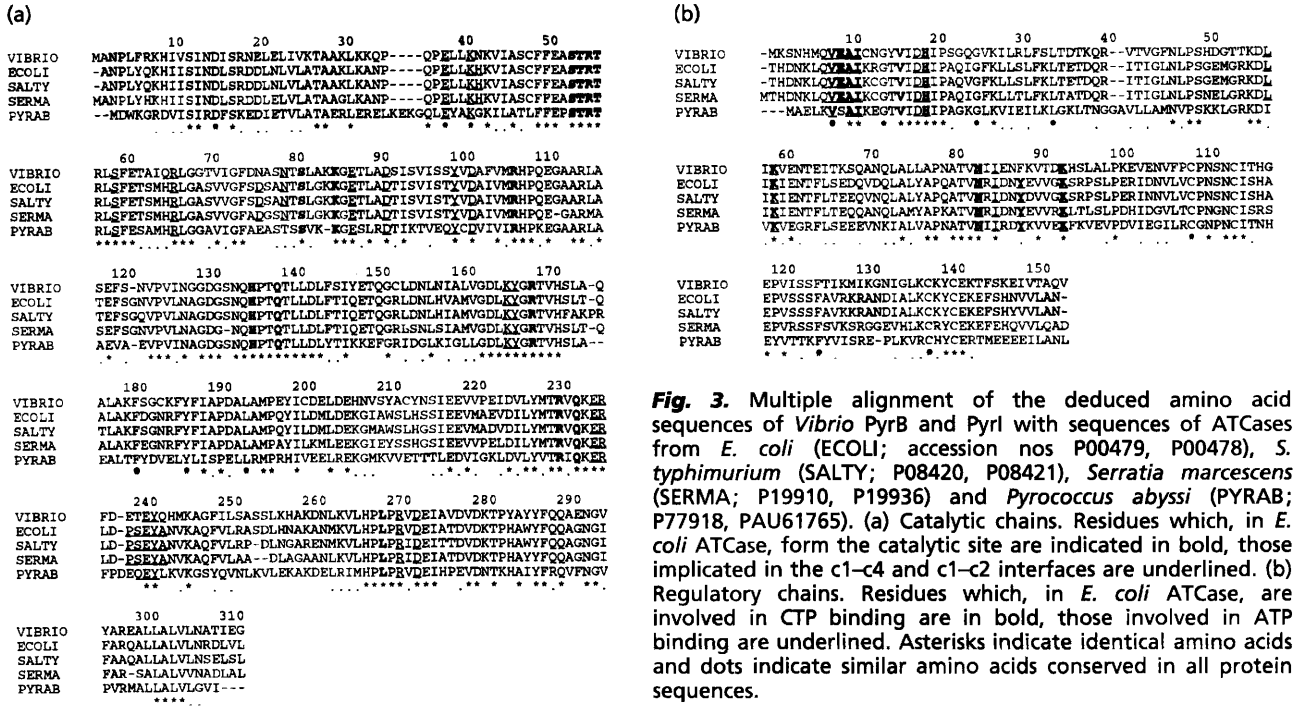

Fig. 3. Multiple alignment of the deduced amino acid sequences of *Vibrio* PyrB and PyrI with sequences of ATCases from *E. coli* (ECOLI; accession nos P00479, P00478), *S. typhimurium* (SALTY; P08420, P08421), *Serratia marcescens* (SERMA; P19910, P19936) and *Pyrococcus abyssi* (PYRAB; P77918, PAU61765). (a) Catalytic chains. Residues which, in *E. coli* ATCase, form the catalytic site are indicated in bold, those implicated in the c1–c4 and c1–c2 interfaces are underlined. (b) Regulatory chains. Residues which, in *E. coli* ATCase, are involved in CTP binding are in bold, those involved in ATP binding are underlined. Asterisks indicate identical amino acids and dots indicate similar amino acids conserved in all protein sequences.

attenuation site: two GC-rich sequences separated by 9 nt, capable of forming a stem–loop structure followed by 7 contiguous T residues. Results obtained with a more distal primer showed that some of the reverse transcripts stop short of this putative stem–loop, suggesting that this structure may actually impede the progress of the transcriptase (not shown). The leader ORF contains a stretch composed almost exclusively of pyrimidines (from nt 48–63). A similar configuration of sequence elements was found in front of the *E. coli* and *Salmonella typhimurium pyrBI* operons (Roof *et al.*, 1982; Michaels *et al.*, 1987). It was originally suggested (Roof *et al.*, 1982) and later established (Liu *et al.*, 1989) that under conditions of pyrimidine starvation, RNA polymerase would pause at the pyrimidine-rich stretch; this would allow the ribosome translating the leader sequence to catch up with the polymerase and therefore prevent the formation of the attenuator structure when the tandem ribosome polymerase moves further downstream. Whether such regulation also operates in *Vibrio* is still unknown. However, it is worth emphasizing that, irrespective of possible regulation, the *Vibrio* control region is functional as far as gene expression is concerned since the primer extension data map the transcription start point at the onset of this region.

The *Vibrio pyrB* and *pyrI* genes are practically adjacent and appear to constitute an operon; only 4 bp separates the two coding regions. The *pyrB* ORF has a coding potential of 310 aa with a predicted molecular mass of 34·4 kDa; *pyrI* would encode a 153 aa polypeptide of 16·8 kDa. The G+C contents of *pyrB* and *pyrI* are 40 and 35 mol% respectively, with a mean of 38% for the whole operon. These values are in agreement with the G+C contents of five arginine genes from the same strain (Z. Liang & Y. Xu, unpublished data).

Analysis of the sequence

The amino acid sequence of *Vibrio* and *E. coli* PyrB polypeptides are 74% identical. Given this high identity, the catalytic (c) chains of *E. coli* and *Vibrio* ATCases are likely to present closely comparable tertiary structures. The majority of the changes (52 of 81, 53 of them being conservative) are localized in the so-called equatorial (or aspartate-binding) domain. In multiple amino acid sequence alignments, the active site residues that contact CP or aspartate in *E. coli* ATCase appear integrally conserved (Fig. 3; Lipscomb, 1994). Moreover, most of the residues which, in *E. coli*, are involved in interactions at the c1–c2 interface (i.e. between subunits of the same catalytic trimer) are conserved. The same is true for residues interacting at the c1–c4 interface (i.e. between subunits belonging to different catalytic trimers). In the *pyrI* subunit, which presents 56% similarity with the *E. coli* counterpart, the nucleotide effector binding site is almost completely conserved: of the 9 residues involved, tyrosine-88 is replaced by phenylalanine and arginine-95 by serine (Fig. 3; Lipscomb, 1994). The four cysteine residues involved in binding to the Zn^{2+} ion in *E. coli* ATCase are conserved.

Properties of *Vibrio* ATCase

The organization of the ATCase genes in *Vibrio* and their comparative sequence analysis strongly suggest that *Vibrio* and *E. coli* ATCase have the same quaternary structure. The molecular mass estimated by filtration through Superose P12 is in keeping with this inference: 320 ± 20 kDa, thus compatible with a 2 (c3) 3 (r2) architecture since the sequence data would predict in that case a mass of 308 kDa. The approximate value of 300 kDa reported for *Vibrio natriegens* (Kenny *et al.*,

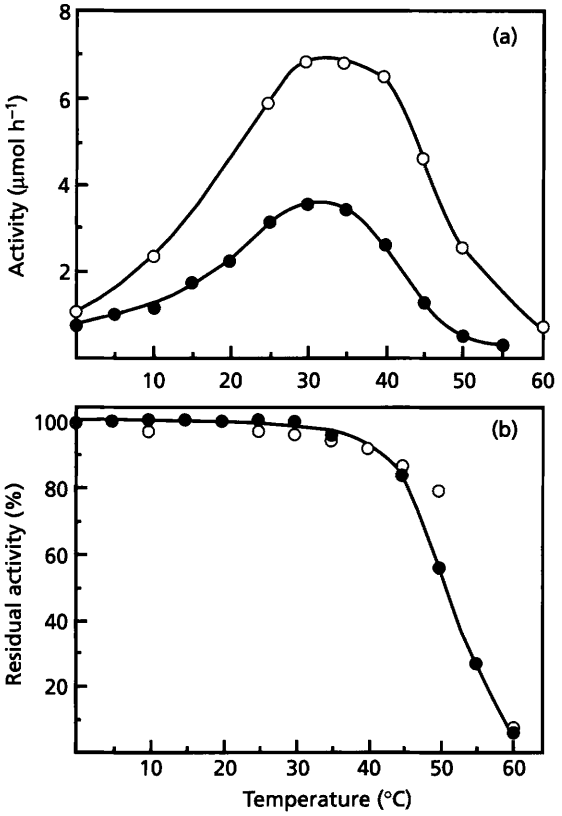

Fig. 4. Effect of temperature on activity (a) and stability (b) of *Vibrio* ATCase in a *Vibrio* extract (●) and in a recombinant *E. coli* extract (○). (a) The temperature dependence of ATCase was determined with the standard assay (see Methods) performed at various temperatures. (b) The thermal stability was determined by measuring residual activity with the standard assay after a 15 min pre-incubation at various temperatures. Data presented are means of duplicate measurements; SEM is less than 10%. In (b) 100% corresponds to 3·6 μmol h^{-1} for the *Vibrio* extract (●) and 5·7 μmol h^{-1} for the recombinant *E. coli* extract (○).

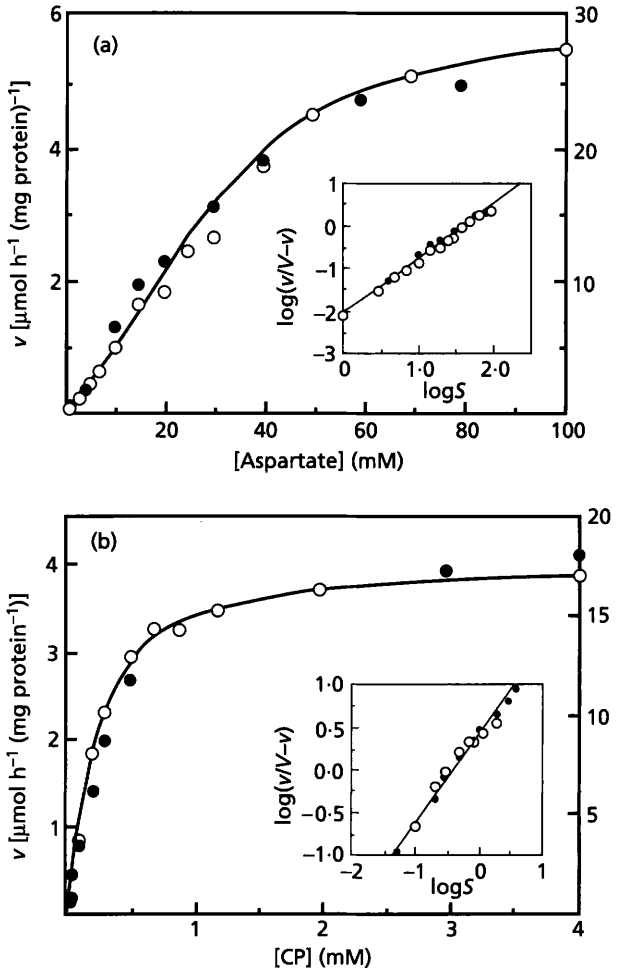

Fig. 5. Saturation curves of *Vibrio* ATCase by aspartate (a) and CP (b). ATCase activity was measured as described in Methods in the presence of increasing concentrations of aspartate or CP. Fixed CP and aspartate concentrations were 5 mM and 20 mM, respectively. Left y-axis, activity in a *Vibrio* extract (●); right y-axis, activity in a recombinant *E. coli* extract (○). The inserts show the corresponding Hill plots. Data presented are means of duplicate measurements; SEM is less than 5%.

1996) corroborates this estimation. The data thus reinforce the notion that in bacteria, class B ATCases are characteristic of the species clustered in the γ-3 group of Gram-negative Proteobacteria (Kenny *et al.*, 1996). It is therefore striking that class B ATCases also appear to be characteristic of Archaea (Bult *et al.*, 1996; Purcarea *et al.*, 1997; Durbecq *et al.*, 1997). A thorough discussion of evolutionary relationships between carbamoyltransferases is presented in Labédan *et al.* (1998).

The study of the pH response suggests that *Vibrio* ATCase activity reaches a maximum at pH 9–10.

The temperature dependence profile of *Vibrio* ATCase (either native or recombinant) indicates an apparent optimum between 30 and 35 °C (Fig. 4a). Psychrophilic enzymes quite commonly display activity at temperatures much higher than the growth optimum of their host (see examples in Morita, 1975, 1992; Russell, 1990, 1992; Davail *et al.*, 1994; Feller *et al.*, 1996). From the physiological point of view, however, it seems relevant that *Vibrio* ATCase is still considerably active in the actual temperature range (up to 14 °C) of its host: at 2 °C, activity approaches 20% of the optimum and 30% at 6 °C. An Arrhenius plot of the data gave an activation energy (E_a) of 41·6 kJ mol^{-1}. By comparison, ATCase from *Pyrococcus abyssi*, a hyperthermophilic archaeobacterium, has an E_a of 65·6 kJ mol^{-1} in the 20–50 °C range but this shifts to 40·9 kJ mol^{-1} in the 50–70 °C range (Purcarea *et al.*, 1994).

Thermostability profiles also proved indistinguishable for native and recombinant ATCase (Fig. 4b); 15 min incubation at 60 °C inactivated the enzyme more than 90%. Half-lives were about 4 min at this temperature and 8 min at 50 °C (data not shown). *Vibrio* ATCase is thus considerably more labile than *E. coli* ATCase which loses only 25% of its activity after 6 h incubation at 60 °C (Kerbiriou & Hervé, 1972). It is therefore interesting to examine whether the differences in amino

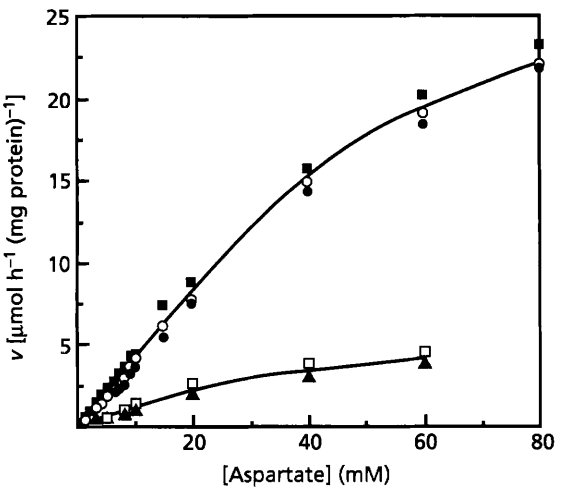

Fig. 6. Influence of NTPs on the aspartate saturation curve of *Vibrio* ATCase produced in *E. coli*. ATCase activity was measured as described in Methods in the presence of increasing concentrations of aspartate without effector (○) or with 2 mM UTP (●), 5 mM ATP (■), 2 mM CTP (□) or 2 mM CTP+2 mM UTP (▲). Data presented are means of at least two separate experiments; SEM is less than 5%.

acid composition already noted between *E. coli* ATCase and its hyperthermophilic homologue from *P. abyssi* also exist in *Vibrio* ATCase and whether they are not accentuated in this enzyme. The most notable features of the mesophilic ATCase with respect to the thermophilic one were a lower number of charged residues likely to participate in stabilizing interactions (arginine, glutamate and lysine), a higher number of the thermolabile residues asparagine and glutamine and a higher number of serine residues (Purcarea *et al.*, 1997). The same trend is observed in *Vibrio* ATCase but, with respect to *E. coli*, the arginine content (and also the R/R+K ratio) undergoes a further and remarkable decrease (14 residues instead of 23), whereas the number of cysteine residues, susceptible to enhanced oxidation at high temperature, rises from 5 to 10. This is a striking increase indeed, considering that four of these cysteines are already involved in the binding of one atom of Zn in the r chain and are expected to be conserved in all class B ATCases. On the whole, therefore, *Vibrio* ATCase presents characteristics which are in keeping with its psychrophilic character.

The response to substrates and effectors was investigated at 30 °C. The aspartate saturation curves suggest homotropic interactions with respect to this substrate (n_H = 1·3±0·05), with a $S_{0.5}$ of 40±4 mM (Fig. 5a). The curves obtained for CP were hyperbolic, with an apparent K_m of 0·3±0·02 mM (Fig. 5b).

The analysis of the effect of nucleotides (see Fig. 6 for data on the recombinant enzyme) showed inhibition by CTP, while ATP and UTP had no effect and no CTP/UTP synergy was observed; the response of the native enzyme was not significantly different (data not shown). The response of the native enzyme to ATP and CTP was also investigated at 15 °C and again no significant differences were noted; moreover AMP, GMP, UMP and GTP were without significant effect at the concentration tested (5 mM), whereas succinate (an analogue of aspartate) and phenylglyoxal were inhibitory (data not shown). The response of *Vibrio* ATCase to various ligands thus resembles that of *E. coli* ATCase except for the lack of activation by ATP and the absence of CTP/UTP synergy. Since the nucleotide binding site of the enzyme is highly conserved (including residues aspartate-19 and leucine-58, both specific for ATP binding in *E. coli* ATCase), it is likely that the insensitivity to ATP is due to substitutions in residues playing a role in the transmission of the regulatory signal.

Conclusions

The temperature response of *Vibrio* ATCase, the marked differences observed between its amino acid composition and that of mesophilic and thermophilic homologues and its close structural relationship to the *E. coli* paradigm indicate that it is a suitable model for biochemical studies on structure–function relationships in a 'cold' allosteric enzyme.

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