The catalytic and regulatory properties of aspartate transcarbamoylase from *Pyrococcus abyssi*, a new deep-sea hyperthermophilic archaeobacterium

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The catalytic and regulatory properties of aspartate transcarbamoylase from *Pyrococcus abyssi* were studied in the GE5 strain isolated from a deep-sea hydrothermal vent located in the North-Fiji Basin in the SW Pacific Ocean. The enzyme from this hyperthermophilic archaeobacterium shows homotropic cooperative interactions between catalytic sites for the utilization of its two substrates, carbamoylphosphate and aspartate. The activity of this enzyme is subject to allosteric regulation. It is feed-back inhibited by the end-product cytidine triphosphate independently of temperature. In contrast, its sensitivity to the feed-back inhibitor uridine triphosphate and to the activator adenosine triphosphate disappears at high temperature. The unusual response of this aspartate transcarbamoylase to carbamoylphosphate analogues suggests a particular mode of binding of this substrate to the catalytic site as compared to the homologous enzymes of other organisms. Aspartate transcarbamoylase of *Pyrococcus abyssi* exhibits a remarkable stability towards high temperature and pressure.

Keywords: *Pyrococcus abyssi*, archaeobacteria, hyperthermophily, aspartate transcarbamoylase

INTRODUCTION

Extreme conditions (temperature, pressure, pH, solvent) are increasingly used to study structure–function relationships in biological macromolecules (Lowe *et al.*, 1993). One aim of such studies is to uncover the molecular mechanisms through which living organisms can adapt to extreme environmental conditions. Hyperthermophilic micro-organisms have been particularly closely studied (Brock, 1978; Stetter *et al.*, 1990; Lowe *et al.*, 1993; Adams, 1993). The comparison of primary structures from homologous enzymes of hyperthermophilic and mesophilic bacteria suggests that resistance to high temperature can be the result of very discrete structural changes (Brock, 1978; Argos *et al.*, 1979; Yutani *et al.*, 1982; Imanaka *et al.*, 1986; Ganter & Plückthun, 1990; Ishikawa *et al.*, 1993) and that the increase in thermostability results from diverse mechanisms. The first studies were made on thermophilic and hyperthermophilic bac-

teria (growth temperature above 60 °C) isolated from hot springs located in volcanic areas.

More recently (Corliss & Ballard, 1977), the discovery of deep-sea biotopes located close to hydrothermal vents brought attention to organisms which are living under high hydrostatic pressure. Among these organisms there are hyperthermophilic bacteria which are adapted to both high pressure and temperature. Many of the bacteria living in these extreme environments are Archaea (previously known as Archaeobacteria) (Woese *et al.*, 1990). In order to understand the mechanisms of their adaptation to these unusual conditions it is necessary to study the structure and the properties of the essential enzymes of these organisms on which their metabolism relies.

One of these micro-organisms, *Pyrococcus abyssi* (strain GE5), was recently isolated from a deep-sea hydrothermal chimney located 2000 m deep in the North-Fiji Basin (SW Pacific) (Auzende *et al.*, 1989; Erauso *et al.*, 1992). This strain was characterized as an obligate anaerobe hyperthermophilic archaeobacterium belonging to the sulphur-metabolizing group. At atmospheric pressure its optimum temperature growth is 96 °C, with a doubling time of

Abbreviations: ATCase, aspartate transcarbamoylase; CPSase, carbamoylphosphate synthetase; CP, carbamoylphosphate; PALA, N-(phosphonacetyl)-L-aspartate.

33 min. *P. abyssi* is barotolerant to barophilic; its growth is stimulated under high hydrostatic pressure, showing adaptation to these conditions (Erauso *et al.*, 1993).

A problem related to cell growth at high temperature is the instability of some intermediary metabolites. This is the case of carbamoylphosphate (CP) which is necessary for the biosynthesis of pyrimidine nucleotides and arginine. In the pyrimidine nucleotides pathway carbamoylphosphate is a substrate of aspartate transcarbamoylase (ATCase), the first enzyme unique to this pathway. This enzyme plays an important role in metabolic regulation and ATCase from *Escherichia coli* is extensively used as a model system to study the molecular mechanisms of cooperativity, allostery and intramolecular signal transmission (Allewell, 1989; Hervé, 1989; Kantrowitz & Lipscomb, 1990; Lipscomb, 1992).

Aspartate transcarbamoylase and carbamoylphosphate synthetase have been studied in various organisms, from bacteria to mammals, and these enzymes allow interesting investigations concerning molecular evolution of protein structure and allosteric regulation (Davidson *et al.*, 1993; Hervé *et al.*, 1993). In view of these interests, the catalytic and regulatory properties of ATCase were studied in the GE5 strain of *P. abyssi*. The partial purification of this enzyme has led to uncontrolled alterations of its structure and regulatory properties. Here we describe a study of its properties in dialysed cell-free extracts. The results show that ATCase from *P. abyssi* exhibits both homotropic cooperative interactions between catalytic sites and allosteric regulation. Its activity is highly resistant to both high temperature and pressure.

METHODS

Chemicals. Carbamoylphosphate (lithium salt), L-aspartate, succinate (sodium salt), phosphonacetate (sodium salt), pyrophosphate, diethanolamine (DEA), N-ethylmorpholine (NEM), 2-(N-morpholino)-ethane sulphonic acid (MES), adenosine triphosphate (sodium salt), cytidine triphosphate (sodium salt) and guanosine 5'-triphosphate, type I (sodium salt) were purchased from Sigma; Tris was from Prolabo; Titriplex III (EDTA) from Merck; uridine triphosphate (sodium salt) from Pharmacia; 2-mercaptoethanol and antipyrine (1,5-dimethyl-2-phenyl-3-pyrazolone) from Kodak; diacetyl monoxime (2,3-butanedione monoxime) from Aldrich; and [U-¹⁴C]aspartate (300 mCi mmol⁻¹; 11.1 GBq mmol⁻¹) from CEA-Saclay. N-(Phosphonacetyl)-L-aspartate (PALA) was a generous gift from Drs V. Narayanan and L. Kedda of the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NIH, Silver Spring, MD, USA.

Cell culture. *P. abyssi* strain GE5 (CNCM I-1302) was grown under anaerobic conditions, at 95 °C, in an artificial sea water medium containing elemental sulfur (S⁰) and supplemented with bactopectone and yeast extract (YPS) (Erauso *et al.*, 1993). The wet weight yield obtained was about 2 g l⁻¹.

Preparation of dialysed cell-free extracts. Cells frozen at -80 °C were suspended into TBE buffer (containing Tris/HCl, pH 8, 50 mM; 2-mercaptoethanol, 1 mM; EDTA, 0.1 mM) and disrupted by sonication six times for 30 s using a Biosonik III sonicator. The extracts obtained were centrifuged at 7000 g and the supernatant was dialysed against the same buffer overnight,

at 4 °C, to eliminate small metabolites, especially nucleotide derivatives, which might interfere with the ATCase activity.

Enzyme assay. Aspartate transcarbamoylase activity was measured by the radioactive test using [¹⁴C]aspartate (Perbal & Hervé, 1972). The ATCase specific activity of dialysed extracts is expressed in units (mg protein)⁻¹. One unit (U) is defined as µmol carbamoylaspartate formed h⁻¹. The standard conditions used were 20 mM aspartate, 5 mM carbamoylphosphate and 50 mM Tris/HCl, at pH 8 and incubation at 37 °C, unless otherwise indicated. When the incubations were performed at 90 °C, the carbamoylphosphate concentration was 40 mM.

Influence of the allosteric effectors. Nucleoside triphosphate (ATP, CTP, UTP, GTP) solutions were buffered at pH 8. Their influence on the rate of the ATCase reaction was measured as previously described (Kerbiriou & Hervé, 1972) in the presence of 3 mM aspartate (Thiry & Hervé, 1978) and the percentage of stimulation or inhibition is expressed as:

$$\% \text{ activation} = \frac{V_a - V_0}{V_0} \times 100$$

$$\% \text{ inhibition} = \frac{V_0 - V_i}{V_0} \times 100$$

where V_0 is the reaction rate in the absence of nucleotides while V_a and V_i are the reaction rates in the presence of activator and inhibitor, respectively.

pH-dependence assay. The buffer system used to determine the pH-dependence of the activity is composed of 0.051 M DEA, 0.051 M NEM and 0.1 M MES adjusted at different pH between 6 and 10 (Leger & Hervé, 1988), using a Knick 655 pH meter and an Ingold microelectrode. Adjustments were made at 37 °C.

Pressure assay. Hydrostatic pressure tests were performed in a high-pressure reactor that allows injection, mixing and sampling at constant pressure (Hui Bon Hoa *et al.*, 1990). When the apparatus was used to follow the rate of reaction as a function of pressure, 300 µl samples were collected in 0.7 ml 0.2 M acetic acid to stop the enzymic reaction. The amount of carbamoylaspartate formed was then determined by the colorimetric test (Prescott & Jones, 1969).

Protein assay. Protein was determined by the Lowry method, using bovine serum albumin as standard.

Data processing. All the kinetic data were treated with the KaleidaGraph program on a Macintosh computer. The substrate saturation curves were fit either to the Hill equation or to the Michaelis-Menten curves.

RESULTS

ATCase activity of *P. abyssi* cell-free extracts

An ATCase activity can be measured in the dialysed cell-free extract of the GE5 strain of *P. abyssi*. In consideration of the thermal lability of the substrate carbamoylphosphate (CP) and of some other metabolites used in this study, this activity was characterized at 37 °C. Furthermore, when possible, the most important properties of the enzyme were subsequently investigated at 90 °C. The ATCase specific activity of these extracts varied by a factor of five from one culture to another, with an average of 2.5 U mg⁻¹ at 37 °C and 55.8 U mg⁻¹ at 90 °C. Since the enzymic test of activity used in most of this study is based on the disappearance of the positive charge of the amino group of aspartate, it was verified that the presence

Fig. 1. Aspartate saturation curve of *P. abyssi* ATCase. ATCase activity was measured as described in Methods in the presence of increasing concentrations of aspartate. (a) Activity at 37 °C using 68 µg protein. (b) Activity at 90 °C using 215 µg protein. The inserts show the corresponding Eadie plots.

of the other substrate, CP, is necessary for the reaction to occur. Indeed, in the absence of CP, the measured ATCase activity was reduced by 99 %. Under the conditions used, the rate of reaction was constant over at least 60 min at 37 °C and 5 min at 90 °C. This reaction rate was proportional to the amount of dialysed cell-free extract up to at least 1150 µg total protein.

A 15-fold purification of ATCase from *P. abyssi* could be obtained by ion-exchange and exclusion chromatography. However, this procedure led to uncontrolled modifications of the molecular form of the enzyme accompanied by loss of its regulatory properties. Consequently the catalytic and regulatory properties of the enzyme were determined in the dialysed cell-free extracts, in which the enzyme shows stable properties.

Catalytic properties of *P. abyssi* ATCase

Aspartate and CP saturation curves. In the presence of a saturating concentration of CP, the aspartate saturation curve of *P. abyssi* ATCase was sigmoidal at both 37 °C and 90 °C, suggesting the existence of homotropic cooperative interactions between catalytic sites (Fig. 1). The kinetic parameters resulting from the fit of a series of such

Fig. 2. CP saturation curve of *P. abyssi* ATCase. ATCase activity was measured as described in Methods at 37 °C in the presence of increasing concentrations of CP. (a) Activity in the presence of 1 mM aspartate, using 278 µg protein. (b) Activity in the presence of 20 mM aspartate, using 106 µg protein. The inserts show the corresponding Eadie plots. In (a) the standard deviation was obtained from the fit to the Hill equation; in (b) the standard deviation was calculated from three identical experimental determinations.

saturation curves to the Hill equation were $S_{0.5} = 3.0 \pm 0.2$ mM aspartate and $n_H = 2.2 \pm 0.2$ at 37 °C, values and standard deviations calculated from six determinations. At 90 °C these parameters were $S_{0.5} = 5.6 \pm 0.3$ mM aspartate and $n_H = 1.8 \pm 0.1$.

CP saturation curves were determined at both low (1 mM) and high (20 mM) aspartate concentrations, at 37 °C (Fig. 2). This procedure was used taking into account the behaviour of *E. coli* ATCase. This homologous enzyme operates through an ordered mechanism in which CP binds first, followed by aspartate (Porter *et al.*, 1969; Schaffer & Stark, 1972; Wedler & Gasser, 1974; Issaly *et al.*, 1982; Hsuanyu & Wedler, 1987; Parmentier *et al.*, 1992). As a consequence, the CP saturation curve is sigmoidal only in the presence of a high concentration of aspartate and the apparent cooperativity for CP reflects only that for aspartate (England *et al.*, 1994). In contrast, in the case of *P. abyssi* ATCase, the CP saturation curve is sigmoidal in the presence of both 1 mM and 20 mM

Fig. 3. Influence of succinate and PALA on the rate of reaction at low concentration of aspartate. ATCase activity was measured as described in Methods but in the presence of 2 mM aspartate. (a) Activity at increasing concentrations of succinate. (b) Activity at increasing concentrations of PALA.

aspartate, the Hill numbers obtained being 1.9 ± 0.2 and 1.8 ± 0.2 respectively. The fit of the saturation curves to the Hill equation gives an $S_{0.5}$ value of $23.4 \pm 3.2 \mu\text{M}$ CP in the presence of 1 mM aspartate and $60.0 \pm 2.3 \mu\text{M}$ CP in the presence of 20 mM aspartate, values and standard deviations calculated from three determinations. In the case of both substrates, the sigmoidicity of the saturation curves is confirmed by the curvature of Eadie plots (inserts of Figs 1 and 2).

Influence of substrate analogues. It is a general property of enzymes showing cooperativity between catalytic sites that some substrate analogues, which are efficient competitive inhibitors, are able to promote the quaternary structure transition which accounts for these interactions. Such is the case of *E. coli* ATCase. The consequence of this phenomenon is that in the presence of a low concentration of aspartate, succinate and other analogues of this substrate increase the rate of the reaction through promoting the transition to the high affinity R state (Collins & Stark, 1971; Jacobson & Stark, 1973). This kind of experiment was performed in the case of *P. abyssi* ATCase using analogues of the two substrates. Figure 3(a) shows that in the presence of 2 mM aspartate low

concentrations of succinate increased the rate of the enzyme reaction by 35%. As expected, higher concentrations of succinate provoked competitive inhibition. This behaviour is identical to that of *E. coli* ATCase, further substantiating the conclusion that *P. abyssi* ATCase shows homotropic cooperative interactions between catalytic sites for the utilization of aspartate.

Since *P. abyssi* ATCase apparently exhibits cooperative interactions for the utilization of CP, the same kind of experiments were performed using analogues of this substrate, which have been shown to be competitive inhibitors of *E. coli* and *Saccharomyces cerevisiae* ATCases (Porter *et al.*, 1969; Penverne & Hervé, 1983). Surprisingly, neither phosphonacetate nor pyrophosphate or phosphate had any influence on the reaction catalysed by *P. abyssi* ATCase, even at a concentration of 10 mM in the presence of 30 μM CP. This result was unexpected, since the K_i of these two compounds for the *E. coli* enzyme are 0.32 mM and 0.09 mM respectively. This lack of inhibition might be related to some particular feature of carbamoylphosphate binding to the catalytic site of *P. abyssi* ATCase. This possibility will be discussed later.

In addition, the bisubstrate analogue PALA, a strong inhibitor of ATCase from different sources (Collins & Stark, 1971; Swyryd *et al.*, 1974; Penverne & Hervé, 1983), also inhibited *P. abyssi* ATCase. At a concentration of 11 μM , PALA provoked 50% inhibition of this enzyme in the presence of 5 mM CP and 20 mM aspartate, with a K_i of 1.1 μM . As in the case of succinate, low concentrations of PALA were able to increase the reaction rate catalysed by the enzyme in the presence of 2 mM aspartate (Fig. 3b). However, the range of PALA concentrations for which such an effect was observed was much wider than in the case of succinate (Fig. 3a), in contrast with what is observed for *E. coli* ATCase (Collins & Stark, 1971; Thiry & Hervé, 1978). The significance of this unexpected observation will be discussed later. The presence of 11 μM PALA abolished the apparent cooperativity for aspartate, the calculated Hill number decreasing from 1.9 ± 0.3 to 1.1 ± 0.2 . This observation is consistent with the results obtained in the presence of succinate, since PALA is also known to promote the T to R transition in *E. coli* ATCase (Collins & Stark, 1971; Moody *et al.*, 1979).

pH-dependence. The influence of pH on the rate of the reaction catalysed by *P. abyssi* ATCase was investigated. Fig. 4 shows that this rate increased uniformly up to around pH 9. Thus it appears that the maximal activity of this enzyme requires a higher pH than that of the homologous ATCase from *E. coli* (Gerhart & Pardee, 1964) and *S. cerevisiae* (Belkaid *et al.*, 1987).

Influence of nucleotides and allosteric regulation

Regulatory properties of *P. abyssi* ATCase were investigated and the results show that the activity of this enzyme is regulated by allosteric effectors differently at low and high temperatures. At 37 °C it is feed-back inhibited by the two end-products CTP and UTP (Fig. 5a). Contrary

Fig. 4. pH-dependence of *P. abyssi* ATCase. ATCase activity was measured at 37 °C as a function of pH as described in Methods in the presence of 5 mM aspartate using 30 µg protein.

Fig. 5. Influence of allosteric effectors on the activity of *P. abyssi* ATCase. ATCase activity was measured in the presence of 3 mM aspartate and increasing concentrations of various nucleotides. (a) Activity at 37 °C. (b) Activity at 90 °C. The percentage inhibition or activation was calculated as described in Methods.

to what is observed in the case of *E. coli* ATCase (Wild *et al.*, 1989), these two effectors do not act in synergy. The influence of increasing concentrations of UTP in the

Fig. 6. Combined effects of the two feed-back inhibitors CTP and UTP. ATCase activity was measured as described for Fig. 5 using increasing concentrations of UTP and in the presence (○) or absence (●) of 1 mM CTP. The percentage inhibition was calculated as described in Methods.

presence of 1 mM CTP (Fig. 6) suggests that the two nucleotides affect the rate of the reaction equally. Their effects are simply additive until the apparent saturation of the regulatory sites. A maximal inhibition of 60% was observed, indicating that these two effectors are not competing with CP at the catalytic site. As in the case of *E. coli* ATCase, ATP is an activator and GTP has no influence on the *P. abyssi* enzyme (Fig. 5a).

Unexpectedly, at 90 °C the activity of this enzyme is regulated only by CTP (Fig. 5b). This feed-back inhibition by CTP was not affected by the presence of 1 mM ATP. It was verified that the lack of influence of UTP and ATP at high temperature was not due to hydrolysis of these two nucleotides or to competition with phosphate which might accumulate as a degradation product of CP.

Influence of temperature

The influence of temperature on the catalytic properties of *P. abyssi* ATCase is of particular interest since the GE5 strain is classified as a hyperthermophilic archaeobacterium (Erauso *et al.*, 1993). In addition, CP, one of the substrates of ATCase, is an unstable chemical (Allen & Jones, 1964) with a half-life of only few seconds at 95 °C. Consequently, temperature effects on the activity of *P. abyssi* ATCase were investigated in different ways.

Thermostability of *P. abyssi* ATCase in absence of substrate.

P. abyssi ATCase samples were preincubated for 15 min at temperatures from 37 °C to 90 °C and the activity was then determined at 37 °C under standard conditions. No inactivation was observed even at the highest temperature. A slight increase in activity was even observed between 37 and 65 °C. On the basis of this result, the stability of the enzyme was examined at 90 °C over a longer period. A sample of *P. abyssi* ATCase was incubated at 90 °C; aliquots were taken at different time intervals and their activity was measured at 37 °C. Under these conditions there was no loss of activity over a period

of 6 h. Taken together, these results show that ATCase from GE5 strain of *P. abyssi* is extremely thermostable, a property that is in accordance with the natural habitat of this micro-organism.

Degradation of CP during the ATCase assay at high temperature. Temperatures higher than 37 °C progressively degrade CP. Therefore, the standard concentration of this substrate (5 mM) might not be saturating the *P. abyssi* enzyme at these higher temperatures, so the kinetic analysis might be inaccurate. Knowing that *E. coli* and *P. abyssi* ATCase have a similar $S_{0.5}$ for CP (60 μ M), the effect of temperature on this substrate was determined by measuring the *E. coli* ATCase activity at 37 °C after 10 min preincubation of the reaction mixture containing 20 mM CP at various temperatures up to 80 °C. In these conditions, the measured reaction rate was constant up to a temperature of 55 °C, showing that this assay can be performed up to that temperature without being hindered by the heat degradation of CP. Consequently, when the experiments were performed at 90 °C, the concentration of CP was 40 mM. Under these conditions the rate of the reaction was constant during 5 min.

Influence of temperature on the reaction rate. The results reported above establish the conditions under which the influence of temperature on the *P. abyssi* ATCase reaction rate can be monitored. In order to determine the influence of temperature on the maximal velocity of this reaction, the aspartate saturation curve of the enzyme was established at different temperatures under the standard conditions described in Methods. As shown in Fig. 7, the rate of the reaction strongly increased, especially above 40 °C, and reached a maximum at 70 °C. Over 70 °C a slight decrease of activity was observed which might be due to chemical carbamylation of ATCase by cyanate, a degradation product of CP. Indeed, preincubation at 90 °C of the enzyme preparation for 5 min provoked such an inactivation, even in the presence of 20 mM glycine as a scavenger. The Arrhenius plot (Fig. 7 insert) is biphasic, indicating an apparent activation energy (E_a) of 65.6 kJ mol⁻¹ over the range 20–50 °C, shifting to 40.9 kJ mol⁻¹ over the range 50–70 °C. From the plot of $\ln(k/T)$ as a function of $1/T$ one can calculate an activation enthalpy $\Delta H^\ddagger$ of 63.0 kJ mol⁻¹ at 37 °C and $\Delta H^\ddagger$ of 38.2 kJ mol⁻¹ at 96 °C and an activation entropy $\Delta S^\ddagger$ of -99.5 kJ mol⁻¹ K⁻¹ at 37 °C and -23.4 kJ mol⁻¹ K⁻¹ at 96 °C.

Influence of pressure

The GE5 strain of *P. abyssi* ATCase was collected from an active chimney at a depth of 2000 m in the Pacific Ocean (Erauso *et al.*, 1993), where the hydrostatic pressure is 20 MPa. In order to determine the influence that pressure could have on the activity of ATCase from this micro-organism, enzyme kinetic experiments under high pressure were performed using the previously described apparatus (Hui Bon Hoa *et al.*, 1990).

In the first kind of experiment, the influence of pressure on the enzyme stability was investigated. Samples were

Fig. 7. Influence of temperature on the rate of the reaction catalysed by *P. abyssi* ATCase. Aspartate saturation curves of *P. abyssi* ATCase were determined at different temperatures under standard conditions as described in Methods and the maximal velocities were calculated. The insert shows the corresponding Arrhenius plot.

Fig. 8. Influence of pressure on the rate of the reaction catalysed by *P. abyssi* ATCase. The rate of reaction was measured as a function of pressure, as described in Methods. Each sample collected contained 108 μ g protein. The insert shows the apparent rate constant of the enzymic reaction as a function of pressure.

preincubated for 15 min under various pressures up to 200 MPa. The initial reaction rate catalysed by these samples when they are brought back to the atmospheric pressure was measured. Up to 200 MPa pressure had no irreversible influence on the catalytic activity of *P. abyssi* ATCase. A slight activation was even observed.

In the second kind of experiment, the influence of pressure on the rate of the reaction catalysed by *P. abyssi* ATCase was directly investigated as described in Methods. As can be seen in Fig. 8, pressure had a negative effect on the reaction rate, with a 50 % decrease observed at 80 MPa.

The plot of $\ln(k)$ against pressure (Fig. 8, insert) indicates an activation volume ($\Delta V^\ddagger$) of 12 ± 0.1 ml mol⁻¹ for this enzymic reaction.

DISCUSSION

Strain GE5 of *P. abyssi* has an aspartate transcarbamoylase (ATCase), the first enzyme of the pyrimidine nucleotide pathway. Partial purification of the enzyme shows variations in its apparent molecular mass, suggesting that this enzyme is oligomeric, as in other organisms. This apparent dissociation is accompanied by an alteration of the regulatory properties of the enzyme.

P. abyssi ATCase shows homotropic cooperative interactions between the catalytic sites for the utilization of the two substrates, CP and aspartate. In the case of *E. coli* ATCase, the cooperativity for aspartate is well documented (Allewell, 1989; Hervé, 1989; Kantrowitz & Lipscomb, 1990; Lipscomb, 1992). This enzyme shows apparent cooperativity for CP only in the presence of a high concentration of aspartate. Taking into account the fact that the reaction catalysed by *E. coli* ATCase proceeds through an ordered mechanism in which CP binds first, followed by aspartate (Porter *et al.*, 1969; Hsuanyu & Wedler, 1987; Parmentier *et al.*, 1992), it was shown that this apparent cooperativity for CP only reflects the cooperativity for the second substrate, aspartate. At low CP concentration the binding of aspartate is restricted and cannot promote the transition to the R form, which has a high affinity for this substrate (England *et al.*, 1994). Such is not the case in *P. abyssi* ATCase since cooperativity is observed at both low and high concentrations of aspartate, presumably when the enzyme is in the two extreme conformations involved in the homotropic cooperative interactions between the catalytic sites for the binding of this substrate.

In the case of aspartate, the existence of cooperative effects is confirmed by the influence of the substrate analogue succinate on the rate of the reaction measured in the presence of a low concentration of aspartate. As in the case of *E. coli* ATCase, the observed increase of the reaction rate is interpreted as resulting from the substrate-analogue-promoted transition of the enzyme from the low-affinity conformation (T state) to the high-affinity conformation (R state). The $S_{0.5}$ value varies from 3.0 ± 0.2 mM aspartate to 5.6 ± 0.3 mM aspartate when the temperature of incubation is raised from 37 °C to 90 °C. Such an effect is expected since the interactions between aspartate and the catalytic site of the enzyme are of an ionic nature (Krause *et al.*, 1987).

Surprisingly, some CP analogues such as pyrophosphate and phosphonacetate, which are good competitive inhibitors of ATCase from other organisms, do not influence the reaction catalysed by *P. abyssi* ATCase. Furthermore, the bisubstrate analogue PALA does not behave similarly towards the *E. coli* and *P. abyssi* enzymes. In the presence of low concentrations of aspartate, maximal stimulation occurs over a much wider PALA concentration range in the case of *P. abyssi* enzyme. Taken

together these results suggest the existence of some differences in the CP binding site between the two enzymes, although they bind this substrate with similar affinities. CP is a very unstable intermediary metabolite whose half-life at 95 °C is only a few seconds. The particular behaviour of the CP binding site of *P. abyssi* enzyme might be related to some adaptive process of channelling of this metabolite from the catalytic site of CP synthetase, where it is synthesized, to the catalytic site of ATCase, where it is used as substrate. Preliminary results obtained in this laboratory suggest that such a process is operating in *P. abyssi*.

The activity of *P. abyssi* ATCase is subject to allosteric regulation, although the nature of this process is temperature dependent. At 37 °C, as in *E. coli* ATCase, ATP acts as an activator and CTP and UTP act as feed-back inhibitors. However, in this archaeobacterium these last two effectors do not act in synergy as they do in the case of the *E. coli* enzyme (Wild *et al.*, 1989; England & Hervé, 1992). The results reported here suggest that the regulatory sites of *P. abyssi* ATCase do not differentiate between the two nucleotides and that the saturation of these sites by one or by the combination of the two effectors leads to a maximal 60 % inhibition. Surprisingly, at 90 °C the activity of *P. abyssi* ATCase is sensitive only to the feed-back inhibitor CTP. Not only is ATP not an activator but it does not alter the response of the enzyme to CTP. This observation suggests that, at high temperature, either ATP does not bind to the enzyme or its regulatory signal is not transmitted.

Although ATCases are generally stable enzymes, that of *P. abyssi* is more resistant to high temperature than the *E. coli* enzyme. Upon incubation at 60 °C for 6 h, *E. coli* ATCase loses 25 % of its activity (Kerbiriou & Hervé, 1972). In contrast, the incubation of *P. abyssi* ATCase for the same period of time at 90 °C does not provoke any decrease of its activity. The determination of the primary structure of this enzyme will provide some information about the amino acid substitutions responsible for this increased thermostability. Temperature increases the maximal rate of the reaction catalysed by the *P. abyssi* enzyme up to 70 °C. The decrease observed above 80 °C is not due to inactivation because, as we have shown, the enzyme is stable at 90 °C. The same phenomenon has been reported in the case of other enzymes from thermophilic and hyperthermophilic archaeobacteria (Breitung *et al.*, 1992; Colombo *et al.*, 1992; Richter & Schafer, 1992; Wakagi *et al.*, 1992). The biphasic nature of the Arrhenius plot suggests that at about 50 °C the enzyme undergoes a structural transition towards a more efficient conformation which catalyses the reaction with a lower energy of activation. A similar behaviour has been observed in the case of D-glyceraldehyde-3-phosphate dehydrogenase from a thermophilic archaeobacterium (Fabry & Hensel, 1987). Interestingly, such a break in the Arrhenius plot has also been reported in the case of *E. coli* ATCase (Wedler & Gasser, 1974), but at a lower temperature. The substantial decrease of the enthalpy of activation and of the negative value of entropy of activation when the temperature is raised from 37 °C to 96 °C indicates that

the transition state of the reaction is reached more easily at high temperature.

The GE5 strain of *P. abyssi* was collected in a deep-sea hydrothermal vent located 2000 m deep in the ocean (Erauso *et al.*, 1992), corresponding to a hydrostatic pressure of 20 MPa. Its growth rate is higher under this pressure (Erauso *et al.*, 1993). The results reported here show that at 20 MPa the rate of the reaction catalysed by *P. abyssi* ATCase is decreased by about 20%, an effect which is fully reversible.

In order to understand the structural features on which the particular properties of ATCase from this hyperthermophilic archaeobacterium are based, the cloning of the gene coding for this enzyme is being undertaken.

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