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Ammonia-dependent synthesis and metabolic channelling of carbamoyl phosphate in the hyperthermophilic archaeon *Pyrococcus furiosus*

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The biosynthesis of carbamoyl phosphate (CP), a metabolic precursor of arginine and the pyrimidines was investigated in the hyperthermophilic archaeon *Pyrococcus furiosus*. The half-life of CP was found to be less than 2 s in the optimum temperature range of this organism (100–102 °C). The carbamoyl-phosphate synthase (CPSase) of *P. furiosus* uses ammonia as the nitrogen donor, and not glutamine like all micro-organisms investigated so far. The M_r of the enzyme, which is devoid of regulatory properties, is 70000, at variance with that of known CPSases. The possible significance of these findings with regard to hyperthermophilic nitrogen metabolism is discussed. Competition experiments with *P. furiosus* crude extracts indicated a marked preference of ornithine carbamoyltransferase (OTCase) for CP synthesized by CPSase rather than for CP added to the reaction mixture. In addition, the bisubstrate analogue δ-*N*-phosphonoacetyl-L-ornithine inhibits the formation of citrulline from bicarbonate, ammonia, ATP and ornithine much less than its synthesis from ornithine and CP in the presence of free OTCase. Such results suggest that, *in vivo*, CPSase and OTCase associate in a complex able to channel CP. Such a channelling may confer protection to CP, thus avoiding the accumulation of toxic amounts of cyanate arising from its decomposition as well as the waste of the two molecules of ATP required for its synthesis.

Keywords: *Pyrococcus furiosus*, ammonia-dependent carbamoyl-phosphate synthase, thermal lability of carbamoyl phosphate, metabolic channelling

INTRODUCTION

Carbamoyl phosphate (CP) is an essential metabolite for both arginine and pyrimidine biosynthetic pathways; ornithine carbamoyltransferase (OTCase) catalyses the formation of citrulline from ornithine and CP in the sixth step of arginine biosynthesis, whereas aspartate carbamoyltransferase (ATCase) promotes the condensation of aspartate and CP into carbamoyl aspartate in the first committed step of pyrimidine biosynthesis. CP is synthesized *in vivo* by a carbamoyl-phosphate synthase (CPSase) which requires bicarbonate, glutamine or ammonia, and MgATP as substrates (Fig. 1).

Abbreviations: ATCase, aspartate carbamoyltransferase; CP, carbamoyl phosphate; CPSase, carbamoyl-phosphate synthase; OTCase, ornithine carbamoyltransferase; PALO, δ-*N*-phosphonoacetyl-L-ornithine.

At least three different types of CPSases have been identified on the basis of their specificity for either ammonia or glutamine as the physiological nitrogen donor, and of their requirement for *N*-acetyl-L-glutamate as cofactor. CPSase I, which requires *N*-acetyl-L-glutamate for maximal activity and does not utilize glutamine in place of ammonia, is found in liver mitochondria and functions in the urea cycle. This enzyme comprises a single polypeptide chain (M_r 155000–165000), which exists either as a dimer or in a monomer–homodimer equilibrium (Elliot & Tipton, 1973; Lusty, 1978; Mori & Cohen, 1978). In contrast to the latter enzyme, CPSase II uses glutamine as the physiologically significant nitrogen donor (Hager & Jones, 1967; Jones, 1980). A first type of CPSase II is present in prokaryotes and simple eukaryotes. These enzymes are dimers comprising a small (M_r 42000) and a large (M_r 120000) subunit. The small subunit catalyses

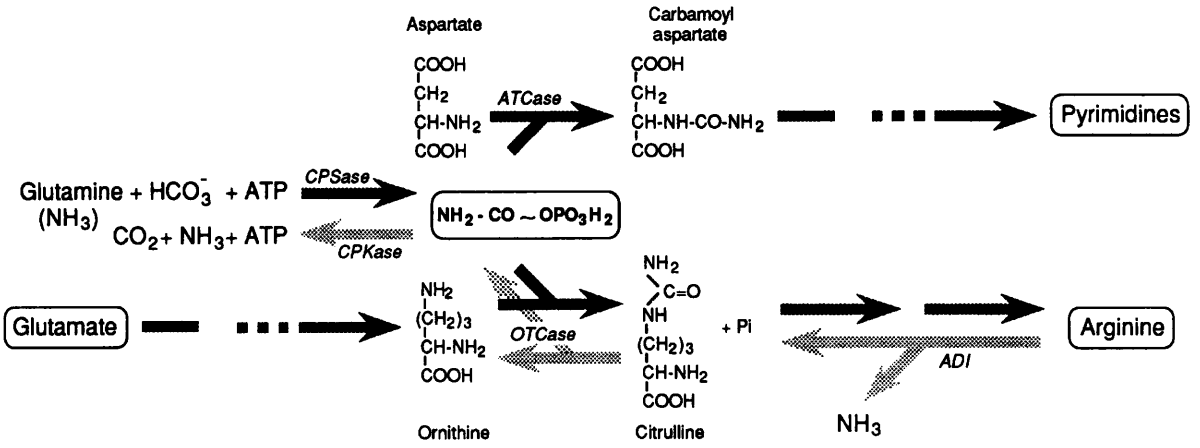

Fig. 1. **Metabolic pathways involving CP. ADI, arginine deiminase; CPKase, carbamate kinase. Dark arrows, biosynthetic pathways; light arrows, catabolic pathway.**

the hydrolysis of glutamine, releasing NH_3 which is transferred to the large subunit; the latter subunit catalyses the formation of CP in a complex reaction which also requires bicarbonate ions, and two MgATP (Meister, 1989). Examples of enzymes in this group include the single CPSase found in enteric bacteria and the arginine-specific CPSases of yeast and *Neurospora* (reviewed by Cunin *et al.*, 1986; Davis, 1986; Meister, 1989). A second type of CPSase II specifically catalyses CP formation required for pyrimidine biosynthesis. These enzymes, which include the CPSase II of higher eukaryotes and the pyrimidine-specific enzymes of yeast and *Neurospora*, are associated as multifunctional complexes with ATCase, or with both ATCase and dihydroorotase (Williams *et al.*, 1970; Coleman *et al.*, 1977; Jones, 1980; Belkaïd *et al.*, 1988). CPSase III, which is present in a number of elasmobranchs and in freshwater teleosts, uses glutamine as the nitrogen substrate and requires *N*-acetyl-L-glutamate as an activator (Anderson, 1980; Anderson, 1981; Casey & Anderson, 1983). The enzyme comprises a single polypeptide chain with a M_r of 155000–165000 and is subject to self-association facilitated by the presence of MgATP, L-glutamine and *N*-acetyl-L-glutamate (Anderson, 1981).

Studies on the decomposition of CP in the mesophilic temperature range (25–37 °C) have shown that CP decomposes readily in aqueous solutions according to different reactions depending on the pH (Allen & Jones, 1964). Acid-catalysed hydrolysis (pH 1–5) leads to the formation of carbon dioxide, ammonia and orthophosphate, whereas a base-catalysed reaction (above pH 6) yields cyanate and orthophosphate (Allen & Jones, 1964). At high temperatures the decomposition of CP could lead to the accumulation of toxic amounts of cyanate, a powerful carbamoylating agent under such conditions. Such a decomposition could also constitute a significant energy waste for the cell since the synthesis of each CP molecule requires two ATP molecules. This led us to propose that, in extreme thermophilic and hyperthermophilic bacteria, the involvement of CP in the biosynthesis of arginine and the pyrimidines depends on mechanisms that protect it against thermal decomposition (Van de Casteele *et al.*, 1990).

It has been recognized that, in many instances, enzymes belonging to the same metabolic pathway are present in multi-enzyme complexes or multifunctional proteins (for reviews see Srere, 1987; Ovádi, 1991; Mathews, 1993). Such complexes might confer a kinetic advantage to the cell by allowing the direct transfer of metabolites between consecutive enzymes, without the need for release into the bulk phase. In extreme thermophiles, the channelling of thermolabile energy-rich intermediates such as CP could conceivably diminish the energy losses resulting from thermal decomposition at high temperature.

In this work we have investigated the kinetics of CP decomposition at high temperatures and indeed found it to be particularly unstable in the temperature range of hyperthermophilic bacteria. We present evidence for CP channelling in a hyperthermophilic archaeon, *Pyrococcus furiosus*, and describe the unusual properties of its CPSase. Part of this work has been published in abstract form (Piérard *et al.*, 1992).

METHODS

Bacterial strain and culture conditions. *Pyrococcus furiosus* Vc1 (DSM 3638) was kindly provided by Professor K. O. Stetter, Regensburg, FRG. Bacteria were grown in a complex medium based on artificial sea-water (Fiala & Stetter, 1986) supplemented with 0·1% yeast extract and 0·5% peptone. Cells were grown at 95 °C in a Biolafite fermenter in 15 l batch runs or in a Braun Biostat U fermenter in 60 l batch runs, with continuous sparging with nitrogen gas at 400 ml min^{-1} to maintain anaerobic conditions and agitation at 200 r.p.m. Cells were harvested by centrifugation at the end of the exponential growth phase (about 2×10^8 cells ml^{-1}) and washed in 3% (w/v) NaCl.

Preparation of cell extracts. Unless otherwise specified, cells were suspended in 50 mM potassium phosphate buffer (pH 7·5) and disrupted by sonication for 5 min in a Heat Systems

ultrasonic oscillator (100 W, 20 kHz). After sonication, the resulting suspension was centrifuged (15 min, 20000 *g*). All these operations were carried out below 10 °C. The supernatant was used for enzyme assays.

Enzyme assays. Carbamoyl-phosphate synthase (CPSase; EC 6.3.5.5) was measured using the coupled assay with OTCase at two temperatures, 60 and 90 °C. The assay at 60 °C was performed with *E. coli* OTCase which proved to be stable at this temperature (Legrain & Stalon, 1976). The reaction mixture contained, in a volume of 1·0 ml, 100 mM Tris/HCl (pH 9·0), 10 mM ATP.Na_2, 10 mM $MgCl_2$, 6 mM L-ornithine, 20 mM [^{14}C]$NaHCO_3$ (0·2 μCi μmol^{-1}), 200 units of partially purified OTCase from *Escherichia coli*, crude extract (0·5–2·0 mg protein) and either 20 mM L-glutamine or 100 mM NH_4Cl. After 10 min incubation, the reaction was stopped by the addition of 1 ml 0·25 M trichloroacetic acid. Excess [^{14}C]$NaHCO_3$ was removed and [^{14}C]citrulline determined as described by Piérard *et al.* (1972). Product formation was a linear function of incubation time up to 20 min and of protein concentration below 2 mg ml^{-1}. The assay at 90 °C was performed in the presence of *P. furiosus* OTCase, using the same reaction mixture and an incubation time of 3 min which did not cause more than 10% loss of [^{14}C]$NaHCO_3$.

Carbamate kinase (EC 2.7.2.2) activity determinations were performed by measurement of the quantity of $^{14}CO_2$ formed in 2·0 ml of a reaction mixture containing 100 mM Tris/HCl buffer (pH 8·0), 10 mM ADP, 10 mM $MgCl_2$, 5 mM [^{14}C]CP (0·1 μCi μmol^{-1}) and extract (up to 15 mg protein). Incubation time was 10 min at 40 °C. Two minutes before the reaction was stopped, 20 μmol ornithine and 400 units of *E. coli* OTCase were added to transform the CP which had not reacted into citrulline. $^{14}CO_2$ was measured as described by Legrain & Stalon (1976). The non-enzymic degradation of CP was measured in control samples without extract.

OTCase (EC 2.1.3.3) was assayed by measuring the formation of citrulline as described by Stalon *et al.* (1972). OTCase activity was measured at 55 °C because of the thermal lability of CP. The reaction mixture contained, in a final volume of 2·0 ml, 200 mM Tris/HCl buffer (pH 7·3 at 25 °C), 2 mM ornithine, 10 mM CP and extract. Unless otherwise specified, the incubation time was 5 min. Formation of citrulline was linear during this time period.

ATCase (EC 2.1.3.2) activity was measured through the formation of carbamoyl aspartate. The reaction mixture contained in a total volume of 0·6 ml, 0·17 M Tris/HCl buffer (pH 8·25), 0–5 units of enzyme, 5 mM CP and 10 mM aspartate (pH 8·25). After 5 min incubation at 55 °C, the reaction was stopped by adding 0·4 ml 5% trichloroacetic acid. Colorimetric measurements of carbamoyl aspartate formed were according to Prescott & Jones (1969).

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.

Partial purification of CPSase. Frozen cells (40 g wet mass) were thawed in 20 ml 50 mM Tris/HCl buffer (pH 7·3) and disrupted by sonication for 10 min in a Heat Systems ultrasonic oscillator (100 W, 20 kHz). Following sonication, the suspension was centrifuged at 20000 *g* for 30 min. The supernatant was collected; the pellet, which consisted of sulfur and cell debris was discarded. Solid ammonium sulfate was added to the supernatant described above to 40% saturation and stirred for 1 h. The solution was centrifuged at 20000 *g* for 30 min. The supernatant was brought to 80% saturation, stirred for 1 h, and then centrifuged at 20000 *g* for 30 min. The pellet was suspended in 50 mM Tris/HCl buffer (pH 7·3) and extensively dialysed against this buffer. The dialysed solution was applied to a DEAE Sepharose CL6B column (1·6 × 25 cm) equilibrated with 50 mM Tris/HCl buffer (pH 7·3). The CPSase activity was eluted with a linear gradient of 0–0·5 M KCl, dissolved in 50 mM Tris/HCl buffer (pH 7·3). Active fractions were pooled and extensively dialysed against 50 mM Tris/HCl buffer (pH 7·3).

Determination of native molecular mass. The native molecular mass of CPSase was determined by measurement of its elution volume on a calibrated Sephadex G-200 SF column (2·5 × 35 cm) which had been equilibrated with potassium phosphate buffer. A 1·2 ml sample was applied, and 1·35 ml fractions were collected at 5 ml h^{-1}. Molecular mass standards used were catalase (M_r 232000), aldolase (158000), albumin (67000), ovalbumin (43000), chymotrypsinogen A (25000) and ribonuclease A (13700).

RESULTS

Thermal decomposition of CP in aqueous solution

The kinetics of CP decomposition was investigated as a function of temperature. Aqueous solutions of CP buffered at pH 8·0, were incubated at various temperatures; after various time intervals, aliquots were withdrawn from these solutions and the residual CP concentration determined enzymically. First-order rate constants (k) were derived from the disappearance of CP; they were plotted as a function of temperature and the best fitting exponential curve was calculated (Fig. 2). Thermal decay was characterized by a Q_{10} of 3. There was no increase in decomposition rate when the ionic strength

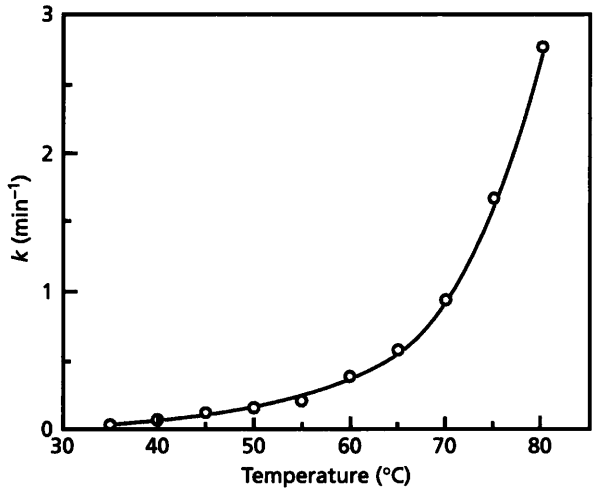

Fig. 2. **Effect of temperature on the rate of CP decomposition. Solutions of CP (10 mM) buffered at pH 8·0 were incubated at the temperatures indicated. At different time intervals, 0·1 ml aliquots were withdrawn from each solution and immediately assayed for residual CP by converting it to citrulline in a reaction mixture containing 100 mM Tris/HCl buffer (pH 8·0), ornithine 20 mM and 500 units *E. coli* OTCase (1 min incubation at 30 °C). Data represent the means from three separate experiments. The values for replicate assays differed from the mean by < 10%. The curve was obtained by exponential fitting of the data points.**

Table 1. Half-life times for the thermal decay of CP in aqueous solution

$t_{1/2}$ values for CP in aqueous solution, 0·15 M Tris/HCl (pH 8·0), were calculated for temperatures between 0 and 100 °C from the k values extrapolated on the curve in Fig. 2 ($t_{1/2} = 0{\cdot}693/k$).

Temperature (°C)	Half-life time ($t_{1/2}$)		
	h	min	s
0	20		
10	7		
20		145	
30		50	
35		30	
40		21	
50		5	
60			105
70			52
80			15
90			5
±100			< 2

of the solution was increased (presence of KCl at concentrations up to 500 mM). There was no noticeable difference between k values obtained at pH 7·5 or pH 9·0 in Tris/HCl buffer (results not shown). Table 1 lists a series of half-life times for CP at pH 8·0, obtained by extrapolation of the curve of Fig. 2. At the optimal growth temperature of *P. furiosus* (100–102 °C; Fiala & Stetter, 1986), the half-life of CP was less than 2 s.

Identification of an ammonia-dependent CPSase activity in cell-free extracts of *P. furiosus*

The failure to detect CPSase activity in cell-free extracts of the archaea *P. furiosus* and *Sulfolobus solfataricus*, where both ATCase and OTCase are present, has been reported previously (Van de Casteele *et al.*, 1990). Since the latter publication, a CPSase activity using both glutamine and ammonia as a nitrogen donor has, however, been detected in *S. solfataricus* extracts (Piérard *et al.*, 1992). In this work, we reinvestigated the synthesis of CP in *P. furiosus* and were able to identify an ammonia-dependent CPSase activity during incubation of extracts of this organism at 60 °C; no activity could be detected when NH_4Cl was replaced by glutamine. This lack of activity was observed at both 60 and 90 °C (Table 2). As the synthesis of CP from ATP, bicarbonate and ammonia could at first sight appear to result from the reverse action of carbamate kinase (an enzyme involved in the deiminase pathway for arginine degradation, see Cunin *et al.*, 1986 and Fig. 1), we assayed crude extracts of *P. furiosus* for its presence, using the technique described in Methods. The failure to detect

Table 2. Substrate specificity of *P. furiosus* CPSase in crude extract or in a partially purified preparation

Activities are the means of at least two independent experiments. The values for replicate assays differed from the mean by < 10% when measured at 60 °C and < 20% when measured at 90 °C. ND, not determined.

Substrate (concentration, mM)	Effector (concentration, mM)	Specific activity [units (mg protein)$^{-1}$]		
		Crude extract (60 °C)	(90 °C)	Partially purified enzyme (60 °C)
NH_4Cl (100); $NaHCO_3$ (20); MgATP (3)	None	3·4	15·4	21·0
Glutamine (50); $NaHCO_3$ (20); MgATP (3)	None	< 0·001	< 0·001	< 0·001
NH_4Cl (100); $NaHCO_3$ (20)	None	< 0·001	ND	ND
NH_4Cl (100); $NaHCO_3$ (20); MgATP (3)	*N*-Acetylglutamate (20)	3·0	14·9	21·6
Glutamine (50); $NaHCO_3$ (20); MgATP (3)	*N*-Acetylglutamate (20)	< 0·001	ND	ND
NH_4Cl (100); $NaHCO_3$ (20); MgATP (3)	UMP (10)	3·2	14·7	20·1
Glutamine (50); $NaHCO_3$ (20); MgATP (3)	UMP (10)	< 0·001	ND	ND
NH_4Cl (100); $NaHCO_3$ (20); MgATP (3)	IMP (10)	3·1	15·3	19·8
Glutamine (50); $NaHCO_3$ (20); MgATP (3)	IMP (10)	< 0·001	ND	ND

Table 3. Partial purification of *P. furiosus* CPSase

Purification step	Total protein (mg)	Total activity (units)	Specific activity [units (mg protein)$^{-1}$]	Yield (%)
Crude extract	3510	12300	3·5	100
$(NH_4)_2SO_4$ fractionation	2400	10660	4·4	87
DEAE Sepharose chromatography	380	7990	21·0	65

any CO_2 evolution upon incubation of crude cell-free extracts with CP and MgADP indicates that the ammonia-dependent CP synthesis is not due to carbamate kinase catalysing the reversible phosphorylation of carbamate by ATP. Moreover, no arginine deiminase activity could be detected in crude cell-free extracts (unpublished results). The CP-catalysing activity of *P. furiosus* extracts can thus be regarded as a CPSase. Partial purification and further characterization of this ammonia-dependent CPSase activity is described below.

Properties of CPSase from *P. furiosus*

Partial purification and molecular mass determination. Table 3 summarizes the partial purification of CPSase from 40 g (wet mass) of frozen *P. furiosus* cells. The preparation obtained after DEAE Sepharose chromatography yielded several bands during gel electrophoresis, but was free of ATCase and OTCase activities. It was used further for kinetic and thermal stability studies.

When this enzyme preparation was submitted to gel filtration on Sephadex G200, the CPSase activity eluted as a single peak at a position corresponding to a M_r of about 70000. Similar results were obtained when a crude extract was submitted to gel filtration on Sephadex G200 (not shown).

Thermal stability. *P. furiosus* CPSase exhibited marked thermal stability. A partially purified preparation obtained after DEAE Sepharose chromatography (Table 3) was incubated for various times (0–120 min) at 100 °C in 20 mM potassium phosphate buffer (pH 7·5). The protein concentration was 10 mg ml^{-1}. These incubations were performed in sealed tubes. The residual CPSase activity of each sample was determined by the standard assay (see Methods). A half-life of 45 min was determined by plotting the logarithm of the residual activity versus the time of preincubation at 100 °C (not shown).

Substrate specificity and kinetic parameters. The most striking property of *P. furiosus* CPSase is its absence of reactivity towards glutamine. In partially purified preparations, as in crude extracts, only ammonia was used as the nitrogen donor in the reaction (Table 2). The values of the apparent K_m for bicarbonate and NH_4Cl, determined using the Lineweaver–Burk and Hanes–Woolfs plots, were $3·8 \pm 0·2$ mM and $0·6 \pm 0·3$ mM, respectively. The effect of MgATP concentration (at equimolar concentrations of ATP and magnesium ions) on the initial rate of the reaction was described by a sigmoid curve with half-maximal activity being observed at a concentration of 0·5 mM. Neither UMP, nor IMP or *N*-acetylglutamate had any effect on the activity of the enzyme at 60 or 90 °C. No evidence was obtained for an effect of triphosphonucleotides.

Competition between CP synthesized by CPSase and exogenous CP in the production of citrulline

The question of CP channelling was addressed by isotopic competition: a crude extract of *P. furiosus* (40 mg protein ml^{-1}) was incubated either at 37 °C or at 55 °C with radioactive bicarbonate in the presence of increasing concentrations of unlabelled CP and the amount of radioactive citrulline formed was then determined as described in Methods. For comparison, an extract of *E. coli* K12 was treated in the same way (at 37 °C only). The results are shown in Fig. 3. After an initial steep decline, indicating the presence of some free OTCase in the extract, the curve almost levelled off; very little competition apparently occurred when the CP concentration was increased further, suggesting the existence of a CPSase/OTCase complex channelling CP rather efficiently. The amount of free OTCase seemed to be higher at 37 °C than at 55 °C, indicating that a higher percentage of this channelling complex may form at higher temperature. Under the same conditions, competition occurred in the *E. coli* extract at the lowest concentrations tested. We also tested the effect of the bisubstrate analogue δ-*N*-phosphonoacetyl-L-ornithine (PALO) in the experiment described in Fig. 3. As expected from the fact that this substance inhibits purified *P. furiosus* OTCase (80% inhibition at 1 μM concentration; C. Legrain, unpublished results), addition of PALO to the system provoked 30–35% inhibition of the amount of citrulline formed in the absence of competing CP, that is by the free OTCase present in the extract. However, at increasing concentrations of CP, PALO displayed only a modest effect (about 15% inhibition, see Fig. 3). This

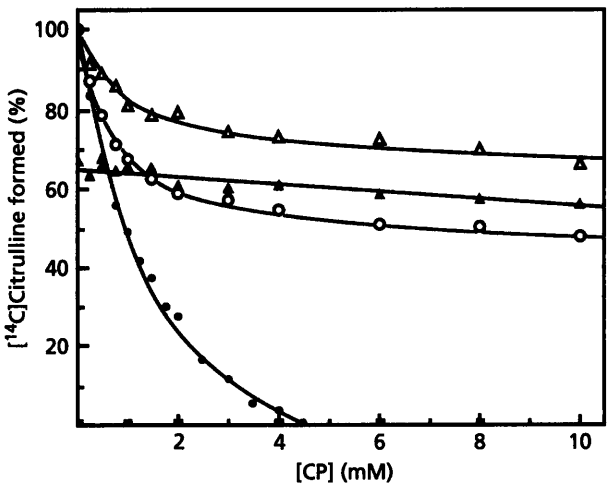

Fig. 3. **Influence of exogenous unlabelled CP on the utilization of labelled CP synthesized by CPSase for citrulline production. Cell-free extracts (40 mg protein ml^{-1}) were incubated in the presence of increasing amounts of unlabelled CP; the reaction was started by adding the CPSase substrates (at saturating level) and [^{14}C]citrulline formed was determined after incubation as described in Methods. [^{14}C]Citrulline formed is expressed as a percentage of the value obtained in the absence of unlabelled exogenous CP and PALO. △, *P. furiosus* extract incubated at 55 °C for 5 min; ○, *P. furiosus* extract incubated at 37 °C for 15 min; ▲, *P. furiosus* extract incubated at 55 °C for 5 min in the presence of 1 mM PALO; ●, *E. coli* extract incubated at 37 °C for 15 min; ■, *E. coli* extract incubated at 37 °C for 15 min in the presence of 1 mM PALO. Each experiment was repeated at least two times without significant differences; results shown are the means of two replicates.**

suggests reduced accessibility of OTCase to PALO in the channelling complex. In contrast, a 100% inhibition of the *E. coli* system by PALO was observed, indicating that such channelling does not occur in this system under the conditions used.

DISCUSSION

Our studies indicate that *P. furiosus* synthesizes CP by a mechanism which appears to be unique among the micro-organisms investigated to date, in that ammonia and not glutamine is the nitrogen donor in the reaction. The enzyme thus behaves as the large subunit of class II CPSases. However, the disparity in M_r (120000 for the large subunit and 70000 for the native *P. furiosus* CPSase) prevents further comparisons in the absence of primary structure data. The *P. furiosus* CPSase gene will have to be cloned and sequenced before proposing possible homologies and structure–function relationships. The novel character of *P. furiosus* CPSase makes it particularly relevant from the point of view of phylogeny, when considering current hypotheses on the possible primeval character of hyperthermophiles.

Our data concerning the kinetics of CP decomposition establish that this intermediate is indeed extremely labile at high temperatures and would therefore appear to require metabolic protection *in vivo*. Experiments described in this paper suggest channelling of CP towards citrulline, a mechanism which could protect CP against thermal decomposition. Competition experiments between labelled CP produced by CPSase and unlabelled CP added to the reaction mixture showed a marked preference of OTCase for the utilization of CP synthesized by CPSase. Since the purification schemes used clearly show that CPSase and the carbamoyltransferases of *P. furiosus* are not integral parts of the same protein, such findings suggest that CPSase and OTCase associate to form a multienzyme complex able to channel this labile intermediate. The fact that the bisubstrate analogue PALO only slightly inhibits the formation of citrulline from bicarbonate, ammonia, ATP and ornithine under the conditions where it inhibits free OTCase, is further evidence for the existence of this complex.

In *Saccharomyces cerevisiae*, channelling of CP has also been observed (Belkaïd *et al.*, 1988) but in this case it is known that CPSase and ATCase activities are carried out by the same polypeptide chain. CP appears to be channelled between CPSase and OTCase in rat liver mitochondria (Cohen *et al.*, 1992). To our knowledge the results presented here are the first indications of CP channelling in prokaryotes. The question whether CP channelling is a prerequisite for arginine and pyrimidine metabolism at high temperature, can be addressed by studying the phenomenon in a variety of phylogenetically unrelated hyperthermophiles.

The question of the non-utilization of glutamine as amino group donor by *Pyrococcus* CPSase is interesting to consider in relation to the hyperthermophilic character of its host. The γ-amido group of glutamine is unstable in hot aqueous solutions and this instability is considerably enhanced in the presence of bicarbonate and phosphate ions (Gilbert *et al.*, 1949), respectively substrate and product of the reaction catalysed by CPSase. As the temperature optimum of *P. furiosus* is between 100 and 102 °C, one therefore wonders whether glutamine would have been an appropriate source of nitrogen for CP synthesis in this and similar organisms. We therefore suggest, as a working hypothesis, that hyperthermophily may be associated with the preferential use of ammonia as nitrogen donor for CP synthesis. Similarly, it may be that other reactions using glutamine as amino group donor in mesophiles will be found to use ammonia in hyperthermophiles. Clearly, several key reactions of biosynthetic metabolism are worth examining in these organisms.

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