Pathways of arginine biosynthesis in extreme thermophilic archaeo- and eubacteria

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The pathway of arginine biosynthesis was investigated in two thermophilic eubacteria, *Thermus aquaticus* and *Thermotoga maritima*, and in two thermophilic archaeobacteria, *Sulfolobus solfataricus* and *Pyrococcus furiosus*. In the first three organisms, arginine biosynthesis proceeds via *N*-acetylated intermediates as in mesophilic micro-organisms. Only the enzymes catalysing the three last steps of the pathway could be detected in *P. furiosus*. The two eubacterial strains possess an ornithine acetyltransferase and are thus able to recycle the acetyl group from acetylornithine to glutamate. The archaeobacterium, *S. solfataricus*, uses the linear pathway in which the formation of ornithine is mediated by the hydrolytic enzyme acetylornithinase. Repression of enzyme synthesis by arginine was observed for most of the enzymes tested in *T. aquaticus* and *S. solfataricus*. Feedback inhibition by arginine was shown only on the ornithine acetyltransferase from *T. aquaticus*. This inhibition pattern is of interest since it would be the first example of control of arginine biosynthesis at this particular step. Data concerning the thermal stability of the arginine biosynthetic enzymes are presented.

Introduction

The biosynthesis of arginine has been extensively investigated in mesophilic micro-organisms (for a review, see Cunin *et al.*, 1986). This biosynthesis proceeds from glutamate in eight enzymic steps: five steps involving *N*-acetylated intermediates lead to ornithine which is converted into arginine through three further steps (Fig. 1). Whereas the intermediates of this sequence are identical in all micro-organisms, there are significant differences with respect to the reaction steps involved and to the regulation of the pathway. Enterobacteriaceae and Bacillaceae use a linear pathway in which the formation of ornithine is mediated by the hydrolytic enzyme acetylornithinase. The other prokaryotes, several methanogenic archaeobacteria (Meile & Leisinger, 1984) and all eukaryotic microbes have been found to recycle the acetyl group by transacetylation of *N*-acetylornithine and glutamate (an energetically more economical version of arginine biosynthesis). The sixth step of arginine biosynthesis, the conversion of ornithine into citrulline, uses carbamoylphosphate, a highly thermolabile precursor (half-life at neutral pH in aqueous solution: about 1 min at 65 °C and <1 s at

100 °C), also required in pyrimidine biosynthesis. The involvement of such a thermolabile intermediate in the metabolism of extreme thermophilic micro-organisms raises the question of the mechanisms used to either bypass carbamoylphosphate or protect it from decomposition at their growth temperatures.

As part of a study of carbamoylphosphate metabolism in thermophilic micro-organisms, we have investigated the pathway through which arginine is synthesized in two thermophilic eubacteria, *Thermus aquaticus* and *Thermotoga maritima*, and two thermophilic archaeobacteria, *Sulfolobus solfataricus* and *Pyrococcus furiosus*. The optimal growth temperature of *T. aquaticus* is 70 °C in minimal medium; the optimal temperatures of *Ta. maritima*, *S. solfataricus* and *P. furiosus* are only available for rich media and are 80, 85 and 100 °C, respectively (Huber *et al.*, 1986; Fiala & Stetter, 1986). Two of these micro-organisms, *Ta. maritima* and *P. furiosus*, are obligate anaerobes. *Ta. maritima* is the most thermophilic eubacterium presently known (Huber *et al.*, 1986). In the phylogenetic tree based upon 16S rRNA sequence comparisons, it represents the oldest and most slowly evolving branch within the eubacteria (Woese, 1987). *S. solfataricus* is a thermoacidophilic archaeobacterium,

while *P. furiosus* belongs to the so-called 'hyperthermophiles' that grow optimally around 100 °C (Stetter, 1986).

Partial results were reported in abstract form in Degryse *et al.* (1976) and Legrain *et al.* (1989).

Methods

Bacterial strains. *Thermus aquaticus* ZO5 was isolated by Degryse *et al.* (1978). *Sulfolobus solfataricus* was obtained from the American Type Culture Collection (ATCC 35091). *Thermotoga maritima* MSB8 and *Pyrococcus furiosus* Vc1 (DSM 3638) were kindly provided by Professor K. O. Stetter, Regensburg, FRG.

Culture conditions. *T. aquaticus* was grown in liquid mineral medium 162 (Degryse *et al.*, 1978) supplemented with 20 mM-sodium pyruvate and 5 mM-(NH₄)₂SO₄. Cultures were grown at 65 °C with shaking at 200 r.p.m. *S. solfataricus* was grown in medium containing 3.1 g KH₂PO₄, 2.5 g (NH₄)₂SO₄, 0.2 g MgSO₄·7H₂O, 0.25 g CaCl₂·2H₂O and either 2 g yeast extract (Difco) or 3 g glucose in 1 litre of distilled water. Cultures were grown at 80 °C with shaking at 200 r.p.m. The anaerobic strains, *Ta. maritima* and *P. furiosus*, were cultivated using the technique of Balch & Wolfe (1976). Cultures (40 ml) were grown in stoppered 120 ml serum bottles pressurized with N₂ (200 kPa) and incubated without agitation. Larger scale cultures were grown in a 15 litre fermentor (Biolafitte) under N₂ (400 ml min⁻¹) with gentle stirring (100 r.p.m.). Bacterial growth was determined by direct count. Unless otherwise specified, *Ta. maritima* was grown at 80 °C in 'MMS'-medium (Huber *et al.*, 1986). *P. furiosus* was cultivated at 95 °C in 'SME'-medium (Stetter *et al.*, 1983; Fiala *et al.*, 1986). Cultures (15 litres) were grown in the absence of elemental sulphur.

Preparation of cell extracts. *T. aquaticus* and *S. solfataricus* cells from exponential phase cultures were harvested by centrifugation (10 min, 7000 g) and washed in 0.9% (w/v) NaCl. *Ta. maritima* and *P. furiosus* were harvested in the late exponential phase (about 2 × 10⁸ cells ml⁻¹) and washed in their respective mineral media. Unless otherwise specified, cells were suspended in 50 mM-Tris/HCl buffer (pH 8.0) 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 done below 10 °C. The supernatant was used for enzyme assays. For arginine inhibition experiments the crude extracts were either dialysed overnight against the extraction buffer or passed through a Sephadex G-25 column.

Enzyme assays. Ornithine carbamoyltransferase activity was measured at 55 °C because of the thermal lability of carbamoylphosphate. Carbamoylphosphate synthetase activity was measured at 60 °C. All the other assays were done at 65 °C for *T. aquaticus* enzymes and at 70 °C for enzymes from the other organisms.

N-Acetylglutamate synthase was measured by a radioassay using L-[¹⁴C]glutamate as substrate. *N*-Acetylglutamate was isolated by high voltage paper electrophoresis. The reaction mixture contained, in a final volume of 50 µl: 100 mM-Tris/HCl buffer (pH 8.0), 20 mM-L-[¹⁴C]glutamate [1 µCi µmol⁻¹ (37 kBq µmol⁻¹)], 2 mM-acetyl-CoA and extract (0.2–0.5 mg protein). The incubation was started by the addition of acetyl-CoA and, after 30 min, terminated by the addition of 10 µl 10% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifugation. Samples (10 µl) were spotted on to Whatman 3MM paper and subjected to high voltage electrophoresis in water/formic acid/acetic acid (910:30:60, by vol.) at 100 V cm⁻¹ for 20 min. The positions of glutamate and *N*-acetylglutamate on the electrophoretograms were deduced from those of reference standards. Glutamate was localized by spraying with ninhydrin reagent and *N*-acetylglutamate by

soaking in 0.01% (w/v) bromocresol green in acetone made alkaline with *N*-ethylmorpholine. The sample areas corresponding to *N*-acetylglutamate and glutamate were cut out and the radioactivity measured by liquid scintillation counting in toluene containing 5 g PPO l⁻¹. Formation of *N*-acetylglutamate was a linear function of time up to 40 min incubation.

N-Acetylglutamate 5-phosphotransferase was measured by the ferric chloride method. The incubation mixture consisted of: 200 mM-Tris/HCl buffer (pH 8.0), 40 mM-*N*-acetylglutamate, 40 mM-MgCl₂, 40 mM-ATP, 400 mM-NH₂OH (pH 8.0) and extract (0.1–0.5 mg protein) in a final volume of 0.5 ml. The reaction was started by the addition of *N*-acetylglutamate and terminated by the addition of 1.0 ml of 1.0 M-HCl containing 5% (w/v) FeCl₃·6H₂O and 4% (w/v) trichloroacetic acid. The absorbance of the hydroxamate-Fe³⁺ complex was measured at 500 nm. Formation of *N*-acetylglutamyl 5-hydroxamate was a linear function of time up to 120 min incubation.

Acetylornithinase was measured by the method of Vogel & McLellan (1970). The protein concentration was about 0.2 mg ml⁻¹ in the assay and the incubation time 10 min.

Ornithine acetyltransferase was determined by a radioassay similar to that described for *N*-acetylglutamate synthase, but with *N*-acetylornithine instead of acetyl-CoA. The reaction mixture contained, in a final volume of 50 µl: 100 mM-Tris/HCl (pH 8.0), 40 mM-L-[¹⁴C]glutamate [1 µCi µmol⁻¹ (37 kBq µmol⁻¹)], 20 mM-*N*-acetylornithine and extract (about 0.5 mg protein). The incubation time was 30 min.

Ornithine carbamoyltransferase was assayed by measuring the formation of citrulline as described by Stalon *et al.* (1972). The *S. solfataricus* enzyme was assayed in a reaction mixture containing, in a final volume of 2.0 ml: 80 mM-EDTA/NaOH buffer (pH 7.8), 15 mM-ornithine, 10 mM-carbamoylphosphate and extract. Ornithine carbamoyltransferases from the other organisms were assayed in a reaction mixture containing: 200 mM-Tris/HCl buffer (pH 8.0), 2 mM-ornithine, 10 mM-carbamoylphosphate and extract. Protein concentration was about 0.5 mg ml⁻¹ in the assay and the incubation time 5 min. Formation of citrulline was linear during this time.

Argininosuccinate synthetase was assayed by a modification of the method of Haas *et al.* (1977), using L-[¹⁴C]citrulline as a substrate. L-Argininosuccinate was converted to its anhydride at acidic pH and isolated by high voltage paper electrophoresis. The reaction mixture contained, in a final volume of 50 µl: 60 mM-Tris/HCl (pH 8.7), 10 mM-sodium L-aspartate, 10 mM-sodium ATP, 10 mM-MgCl₂, 2 mM-L-[ureido-¹⁴C]citrulline [3 µCi µmol⁻¹ (111 kBq µmol⁻¹)], extract (0.2–0.5 mg protein) and 200 mM-sodium fumarate. The incubation was terminated by the addition of 20 µl 1.5 M-formic acid. The samples were boiled for 15 min and precipitated protein was removed by centrifugation. Samples (10 µl) were spotted on to Whatman 3MM paper and subjected to high voltage electrophoresis in water/formic acid/acetic acid (910:30:60, by vol.) at 100 V cm⁻¹ for 15 min. The positions of citrulline, argininosuccinate, argininosuccinic anhydride and arginine on the electrophoretograms were deduced from those of reference standards, localized by spraying with ninhydrin reagent. The corresponding sample areas were cut out and the radioactivity measured by liquid scintillation counting in toluene containing 5 g PPO l⁻¹. Formation of argininosuccinate was a linear function of time up to 30 min incubation.

Argininosuccinase was assayed by the procedure of Theil *et al.* (1969). Protein concentration was about 1 mg ml⁻¹ in the assay and the incubation time 20 min.

Carbamoylphosphate synthetase was measured by the method of Piérard *et al.* (1972). The reaction mixture contained, in a volume of 1.0 ml: 100 mM-Tris/HCl (pH 9.0), 10 mM-sodium ATP, 10 mM-MgCl₂, 6 mM-L-ornithine, 20 mM-NaH¹⁴CO₃ [0.2 µCi µmol⁻¹ (7.4 kBq µmol⁻¹)], 200 units of partially purified ornithine carbamoyltransferase from *E. coli*, crude extract (0.5–2.0 mg protein) and either

20 mM-L-glutamine or 100 mM-NH₄Cl. The reaction was stopped by the addition of 1 ml 0.25 M-trichloroacetic acid. Excess NaH¹⁴CO₃ was removed and [¹⁴C]citrulline determined as described by Piérard *et al.* (1972). Enzyme activity was a linear function of incubation time up to 20 min and of protein concentration below 2 mg ml⁻¹.

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.

Results

In vitro assay of enzyme activities

N-Acetylglutamate synthase and *N*-acetylglutamate 5-phosphotransferase activities (Fig. 1, steps 1 and 2) were detected in three of the four strains examined (Table 1). The specific activities of these two enzymes were comparable to those reported for mesophilic bacteria (Udaka, 1966; Haas *et al.*, 1972; Leisinger & Haas, 1975; Abdelal & Nainan, 1979). These two activities could not be detected in *P. furiosus* extracts. *N*-Acetylglutamate-5-semialdehyde dehydrogenase and *N*²-acetylornithine 5-aminotransferase activities (Fig. 1, steps 3 and 4), for which no reliable high temperature assays are available, were not measured.

Two enzymes, acetylornithinase (Fig. 1, step 5) and ornithine acetyltransferase (Fig. 1, step 5'), can convert *N*²-acetylornithine to ornithine. Ornithine acetyltransferase activity was found in *T. aquaticus* and *Ta. maritima* extracts, but could not be detected in *S. solfataricus* and *P. furiosus* extracts (Table 1). The *K_m* value for *N*²-acetylornithine at 10 mM-glutamate was 0.5 mM for the *T. aquaticus* enzyme and 2 mM for the *Ta. maritima* enzyme. In *S. solfataricus*, acetylornithine was deacetylated by the hydrolytic enzyme acetylornithinase. A low level of an acetylornithinase with a poor affinity for acetylornithine (*K_m* > 40 mM) was found in *T. aquaticus* extracts. A very small amount of acetylornithinase activity was also detected in *Ta. maritima*. The latter two activities probably result from deacetylation of ornithine by non-specific enzymes (see Discussion).

The last three enzymes of arginine biosynthesis, ornithine carbamoyltransferase, argininosuccinate synthetase and argininosuccinase, were detected in the four strains examined (Table 1). The high levels of ornithine carbamoyltransferase activity measured in the two archaeobacteria were comparable to those described for methanogenic archaeobacteria (Meile & Leisinger, 1984).

Repression of enzyme formation by arginine

In *T. aquaticus*, the synthesis of all the arginine biosynthetic enzymes, except *N*-acetylglutamate 5-phos-

Fig. 1. Arginine biosynthesis. 1, *N*-Acetylglutamate synthase (acetyl-CoA:L-glutamate *N*-acetyltransferase; EC 2.3.1.1); 2, *N*-acetylglutamate 5-phosphotransferase (ATP:*N*-acetyl-L-glutamate 5-phosphotransferase; EC 2.7.2.8); 3, *N*-acetylglutamate 5-semialdehyde dehydrogenase [*N*-acetyl-L-glutamate-5-semialdehyde:NADP⁺ 5-oxido-reductase (phosphorylating); EC 1.2.1.38]; 4, *N*²-acetylornithine 5-aminotransferase (*N*²-acetyl-L-ornithine:2-oxoglutarate aminotransferase; EC 2.6.1.11); 5, acetylornithinase (*N*²-acetyl-L-ornithine amidohydrolase; EC 3.5.1.16); 5', ornithine acetyltransferase (*N*²-acetyl-L-ornithine:L-glutamate *N*-acetyltransferase; EC 2.3.1.35); 6, ornithine carbamoyltransferase (carbamoylphosphate:L-ornithine carbamoyltransferase; EC 2.1.3.3); 7, argininosuccinate synthetase [L-citrulline:L-aspartate ligase (AMP-forming); EC 6.3.4.5]; 8, argininosuccinase (L-argininosuccinate arginine-lyase; EC 4.3.2.1).

Table 1. Specific activities of arginine biosynthetic enzymes in extreme thermophilic bacteria

Specific activities are means of at least two measurements made on independent cultures. The values for replicate assays differed from the mean by <20% for *N*-acetylglutamate synthase, ornithine acetyl transferase and argininosuccinate synthetase, and by <10% for the other activities. ND, Not determined.

Strain	Addition to minimal growth medium*	Enzyme activity [units (mg protein) ⁻¹]						
		<i>N</i> -Acetylglutamate synthase	<i>N</i> -Acetylglutamate 5-phosphotransferase	Acetylornithinase	Ornithine acetyltransferase	Ornithine carbamoyltransferase	Argininosuccinate synthetase	Argininosuccinase
<i>Thermus aquaticus</i>	pyr, NH ₄ ⁺	0.14	0.45	0.6	0.34	3.5	0.39	1.33
	pyr, NH ₄ ⁺ , arg	0.07	0.46	ND	0.13	0.3	0.07	0.29
<i>Thermotoga maritima</i>	Starch, YE	0.45	0.15	0.02	0.35	9.3	0.041	0.03
	Starch, YE, arg	0.45	0.10	ND	0.35	8.3	0.026	ND
<i>Sulfolobus solfataricus</i>	glc, NH ₄ ⁺	0.11	0.18	7.6	<0.001	15.3	0.040	0.34
	glc, NH ₄ ⁺ , arg	0.10	0.06	7.8	ND	5.0	0.016	0.10
	YE	0.12	0.05	8.9	<0.001	7.2	0.027	0.18
	YE, arg	0.10	0.05	8.0	<0.001	4.3	0.014	0.07
<i>Pyrococcus furiosus</i>	pep, YE	<0.001	<0.02	<0.005	<0.001	26.0	0.014	0.20

* pyr, pyruvate (20 mM); glc, glucose (20 mM); YE, yeast extract (*Ta. maritima*, 0.05%; *S. solfataricus*, 0.2%; *P. furiosus*, 0.1%); pep, peptone (0.5%); arg, arginine (5 mM). The NH₄⁺ concn was 5 mM. The arginine concn in 0.1% YE was about 0.1 mM.

photransferase, was repressed by arginine. The repression ratios ranged from 2 for *N*-acetylglutamate synthase to 10 for ornithine carbamoyltransferase. In *S. solfataricus*, a threefold repression of enzyme formation by arginine was observed, except for *N*-acetylglutamate synthase and acetylornithinase. The effect of arginine on the levels of *Ta. maritima* and *P. furiosus* enzymes could not be tested, since addition of yeast extract to the culture medium was necessary to obtain a reasonable growth yield (about 2×10^8 cells ml⁻¹). However, addition of 5 mM-arginine to a medium containing 0.05% yeast extract (about 0.05 mM-arginine) led to a small but reproducible decrease of the level of *N*-acetylglutamate 5-phosphotransferase and argininosuccinate synthetase from *Ta. maritima*.

Feedback inhibition by arginine

In bacteria which synthesize arginine by the linear pathway, as observed for *S. solfataricus*, *N*-acetylglutamate synthase (Fig. 1, step 1) is the target enzyme for feedback inhibition by arginine (Vyas & Maas, 1963; Udaka, 1966; Abdelal & Nainan, 1979; Cunin *et al.*, 1986). In the organisms which recycle the acetyl group by transacetylation of *N*²-acetylornithine and glutamate, *N*-acetylglutamate synthase fulfils an anaplerotic function, and it is the second enzyme of the pathway, *N*-acetylglutamate 5-phosphotransferase (Fig. 1, step 2), which is feedback inhibited by arginine (Udaka, 1966; Meile & Leisinger, 1984; Cunin *et al.*, 1986).

Inhibition by arginine was tested for all the enzymes

listed in Table 1, except argininosuccinase, by adding L-arginine (concentration range: 0.01–100 mM) to the reaction mixture of the enzyme assays described above. No feedback inhibition could be detected for the first and second enzymes of the three strains which display these activities. However, arginine was found to inhibit the ornithine acetyltransferase activity of *T. aquaticus*. Inhibition was non-competitive towards acetylornithine and the apparent *K*_i was 1.75 mM; 90% inhibition was reached at 10 mM-arginine. The activity of argininosuccinate synthetase of all four strains was also inhibited by arginine. Apparent *K*_i values for arginine of 1.4, 0.42 and 0.25 mM respectively were measured for the argininosuccinate synthetase of *T. aquaticus*, *Ta. maritima* and *S. solfataricus* (not determined for *P. furiosus*); the arginine concentrations for 90% inhibition were 8, 4 and 2 mM. This inhibition is probably due to a competition of arginine with citrulline for the same site on the enzyme as described for yeast argininosuccinate synthetase (Hilger *et al.*, 1979).

Biosynthesis of carbamoylphosphate

Ornithine carbamoyltransferase, which catalyses the sixth step of arginine biosynthesis, needs carbamoylphosphate as second substrate. A carbamoylphosphate synthetase activity, which requires bicarbonate, ATP and glutamine or ammonia as substrates, was found in extracts of the two eubacteria, *T. aquaticus* and *Ta. maritima*. Unlike most of the other prokaryotic carbamoylphosphate synthetases, the specific activity of these

Table 2. Specific activities of extreme thermophilic carbamoylphosphate synthetase with glutamine and NH_4Cl as substrate

Specific activities are means of at least two measurements made on independent cultures. The values for replicate assays differed from the mean by <20%.

Strain	Addition to minimal growth medium*	Specific activity [units (mg protein ⁻¹)]		Apparent K_m (mM)	
		Glutamine	NH_4Cl	Glutamine	NH_4Cl
<i>Thermus aquaticus</i>	pyr, NH_4^+	0.036	0.25	0.2	11
	pyr, NH_4^+ , arg	0.029	0.19		
	pyr, NH_4^+ , ura	0.028	0.13		
	pyr, NH_4^+ , arg, ura	0.026	0.13		
<i>Thermotoga maritima</i>	Starch, YE	0.028	0.07	1.3	9
	Starch, YE, arg	0.028	0.08		

* pyr, pyruvate (20 mM); arg, arginine (5 mM); ura, uracil (25 $\mu\text{g ml}^{-1}$); YE, yeast extract (0.05%). The NH_4^+ concn was 5 mM. K_m values were determined at saturating levels of the other substrates.

enzymes was higher with ammonia than with glutamine as a substrate under our assay conditions (Table 2). However, the affinity for glutamine was higher than for ammonia (Table 2). Addition of arginine or uracil to the growth medium of *T. aquaticus* had little effect on the level of carbamoylphosphate synthetase (Table 2). Cumulative repression by arginine plus uracil, as described for *E. coli* (Piérard *et al.*, 1965), was not observed in *T. aquaticus*. No carbamoylphosphate synthetase activity could be detected in the two archaeobacterial strains. In *S. solfataricus*, the search for carbamoylphosphate synthetase activity was made under various buffer and pH conditions, with glutamine, asparagine or ammonia as substrates, and by coupling the reaction with that of ornithine or aspartate carbamoyltransferase.

Thermal stabilities of some arginine biosynthetic activities

Half-life times were determined by incubating crude extracts for various times at a fixed temperature. The residual activities were measured as described in Methods. Marked stability at high temperature is a characteristic of most of these activities. Their half-life times at 80 °C were greater than 1 h, except for the ornithine carbamoyltransferase activity from *T. aquaticus* ($t_{1/2}$: 30 min at 80 °C and 3 min at 90 °C) and *Ta. maritima* ($t_{1/2}$: 3 min at 80 °C). The two latter activities could, however, be protected from heat denaturation by addition of ornithine and/or phosphate. In contrast, *S. solfataricus* and *P. furiosus* ornithine carbamoyltransferase activities proved highly thermostable ($t_{1/2}$: 10 h at 85 °C for *S. solfataricus*, 3 h at 90 °C and 30 min at 100 °C for *P. furiosus*).

Discussion

In at least three of the thermophilic strains examined here, the biosynthesis of arginine proceeds via *N*-acetylated intermediates as in mesophilic micro-organisms. The two strains of extreme thermophilic eubacteria, *T. aquaticus* and *Ta. maritima*, possess an ornithine acetyltransferase. Owing to the presence of this enzyme they are able to recycle the acetyl group from acetylornithine to glutamate and to save part of the energy required for arginine biosynthesis. An acetylornithinase activity was also detected in these two strains. However, the *T. aquaticus* acetylornithinase activity had poor affinity for *N*-acetylornithine ($K_m > 40$ mM) compared to that of ornithine acetyltransferase for the same substrate ($K_m = 0.5$ mM). In *Ta. maritima*, the acetylornithinase activity detected was very low. These activities have presumably little or no biosynthetic function. It has been shown in yeast that the acetylornithinase measured *in vitro* can be ascribed to a metal-activated carboxypeptidase (Degryse, 1974). Such a carboxypeptidase could be responsible for the acetylornithinase activity measured *in vitro* in *T. aquaticus* and *Ta. maritima* extracts. These activities could also result from the action of broad substrate specificity aminoacylase or dipeptidase such as those described by Cho *et al.* (1987, 1988).

The archaeobacterium *S. solfataricus* lacks ornithine acetyltransferase and thus forms *N*-acetylglutamate exclusively via the energetically less favourable reaction catalysed by *N*-acetylglutamate synthase, investing 1 mol of acetyl CoA per mol of *N*-acetyl intermediate synthesized. In contrast, methanogenic archaeobacteria have been found to use the cyclic pathway of ornithine synthesis (Meile & Leisinger, 1984). These data show that the diversity in the biosynthesis of arginine which

was observed previously among eubacteria also prevails for archaeobacteria.

In *P. furiosus*, none of the enzymes catalysing the steps from glutamate to ornithine could be detected. Since we were unable to grow this bacterium on a defined medium, the requirement for arginine or ornithine could not be tested. *P. furiosus* is an obligate anaerobe and, therefore, we cannot exclude that the failure to detect these enzymes was due to their inactivation by oxygen. However, it has been reported that the enzymes of arginine biosynthesis in obligate anaerobic methanogens are insensitive to oxygen (Meile & Leisinger, 1984).

Repression of enzyme synthesis by arginine was observed for most of the enzymes tested in *T. aquaticus* and *S. solfataricus*. The changes in the levels of enzyme are less pronounced than those generally observed in mesophilic bacteria. Whereas markedly different repression ratios were obtained for the various *T. aquaticus* enzymes, a constant ratio of 3 was measured for the repressible *S. solfataricus* enzymes. Genetic data will be necessary to decide whether this behaviour is an indication of a coordinate repression mechanism similar to those known in mesophilic eubacterial operons.

The pattern of control of enzyme activity previously described, namely feedback inhibition by arginine of acetylglutamate synthase in organisms possessing the linear pathway or of the next enzyme in organisms recycling the acetyl group (Udaka, 1966; Cunin *et al.*, 1986), was not observed in *T. aquaticus*. However, arginine inhibits the ornithine acetyltransferase of this organism. This pattern of inhibition is of interest since, in the cyclic pathway of ornithine synthesis, ornithine acetyltransferase may play the rôle of a key enzymic step determining the flux of arginine precursors (Fig. 1). This would be the first example of control of arginine biosynthesis at this particular step. Whether this inhibition actually plays this regulatory rôle *in vivo* remains to be established, since the inhibition constant of arginine ($K_i = 1.75$ mM) is relatively high as compared to the affinity of the enzyme for *N*-acetylornithine ($K_m = 0.5$ mM). One must, however, take into account that these values were determined with crude extracts and at saturating levels of glutamate. *Ta. maritima* and *S. solfataricus* are apparently devoid of any feedback inhibition by arginine of the early enzymes of the pathway. It will be interesting to determine whether this is a typical feature of extreme thermophiles.

Stability at high temperature is a characteristic of most of the enzymes studied, with the exception of the ornithine carbamoyltransferases of the two eubacterial strains. Yet these activities are stabilized by ornithine and phosphate, as previously observed for *E. coli* ornithine carbamoyltransferase (Legrain & Stalon, 1976). The ornithine carbamoyltransferase from *T.*

aquaticus has been purified to homogeneity. The enzyme consists of a trimer of identical subunits, as are the mesophilic ornithine carbamoyltransferases analysed so far (Van de Castele *et al.*, 1989).

Carbamoylphosphate synthetase activity was only detected in the two eubacterial strains examined. In the archaeobacteria, *S. solfataricus* and *P. furiosus*, we could not detect any carbamoylphosphate synthetase activity, although both aspartate and ornithine carbamoyltransferases were present. How these micro-organisms synthesize carbamoylphosphate remains an open question.

The involvement of carbamoylphosphate in the metabolism of extreme thermophiles raises the problem of avoiding aspecific carbamoylation of amino compounds by cyanate resulting from heat decomposition of carbamoylphosphate. In this respect, a constitutive cyanase activity, found in *T. aquaticus* extracts (unpublished observation), might play a detoxifying function. Another way of avoiding chemical carbamoylation might be to protect carbamoylphosphate from decomposition by channelling it through multi-enzyme complexes comprising the enzymes of carbamoylphosphate synthesis and utilization. Whether metabolic channelling of carbamoylphosphate occurs in extreme thermophilic micro-organisms is under investigation.

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