Enzymes of arginine biosynthesis in methanogenic bacteria[1]

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Summary. Methanobacterium thermoautotrophicum, Methanococcus vannielii and *Methanobrevibacter arboriphilus,* the 3 methanogenic bacteria tested, possessed an ornithine acetyltransferase and an arginine-sensitive *N*-acetylglutamate 5-phopshotransferase. These 2 enzymes, as well as acetylornithinase and ornithine carbamoyltransferase, were insensitive to oxygen and were present at activity levels comparable to the levels observed in eubacteria.

The biosynthesis of arginine in microorganisms proceeds from glutamate via 4 *N*-acetylated intermediates to ornithine, which is converted in 3 further enzymatic steps to arginine. While the intermediates of this biosynthetic sequence are identical in all microorgansims, there are differences with respect to the reaction steps involved and the regulation of the pathway[3]. Most prokaryotes[3] and all eukaryotic microbes[4] that have been examined possess the enzyme ornithine acetyltransferase (N^2-acetyl-L-ornithine: L-glutamate *N*-acetyltransferase; EC 2.3.1.35). Owing to the presence of this enzyme they are able to recycle the acetly group in the pathway from glutamate to ornithine and thus conserve part of the energy required for arginine biosynthesis[5]. In contrast, enterobacteriaceae and *Bacillus* lack ornithine acetyltransferase. They form *N*-acetylglutamate exclusively via the energetically less favorable reaction catalyzed by *N*-acetyl-glutamate synthase (acetyl-CoA: L-glutamate *N*-acetyltransferase: EC 2.3.1.1) investing 1 mol of acetyl CoA per mol of *N*-acetylated intermediate synthesized. With the concept that the archaebacteria form a third kingdom of life[6] the question arises as to the modification of the arginine biosynthetic pathway realized in representatives of this group of organisms. We have therefore assayed some arginine biosynthetic enzymes in methanogenic bacteria and will present evidence which suggests that in the methanogens examined arginine biosynthesis proceeds via the ornithine acetyltransferase pathway. Since methanogens are obligate anaerobes, the effect of oxygen on the catalytic activity of the arginine enzymes under investigation was also examined.

Materials and methods. Methanobacterium (Mb.) *thermoautotrophicum* Marburg (DSM 2133) was obtained from G. Fuchs, Marburg University, FRG and *Methanococcus vannielii* (DSM 1224) from the German Collection of Microorganisms, Göttingen, FRG. They were grown as described[7]. *Methanobrevibacter arboriphilus* strain AZ (DSM 744) was obtained from A. Zehnder, EAWAG, Dübendorf, Switzerland and grown in the same medium and under the same conditions as *Mb. thermoautotrophicum* except that the incubation temperature was 37 °C. Anaerobe facilities and gas processing and the preparation of buffers have already been described[8].

Cells from the late exponential growth phase were transferred inside the anaerobe chamber into centrifuge tubes fitted with air-tight caps. After centrifugation outside the chamber the tubes were reintroduced into the chamber where the supernatant was decanted. The cell paste was either used immediately for the preparation of cell extract or stored at −20 °C under a H_2/CO_2 gas mixture (80%/20%) at 2 bar until used. Crude extracts were prepared by sonication of a 20% (w/v) suspension of wet cells in 0.05 M potassium phosphate (pH 7.0) containing 2 mM dithiothreitol, or by passage through a French pressure cell. Sonication was performed[9] inside the anaerobe chamber. When the French press was used, the pressure cylinder was filled inside the chamber but used for extract preparation at 120 MPa outside the chamber. Freshly prepared extract was collected in a serum flask containing a N_2/H_2 gas mixture (92%/8%). Cell debris was sedimented by centrifugation in air-tight tubes at 40,000 × g for 40 min. The resulting crude extracts contained 8.0–15.0 mg of protein/ml as determined by the method of Lowry et al. For some experiments the crude extracts were desalted by passage through Sephadex G-25 inside the anaerobe chamber.

All reagents for measuring enzyme activities in crude extracts under anaerobic conditions were degassed and preincubated before use during 48 h in the anaerobe chamber under an atmosphere of N_2/H_2 (92%/8%) containing less than 4 ppm of O_2. Immediately before use the solutions were evacuated and gassed 3 times with N_2/H_2. Enzyme assays under aerobic conditions were performed with the same reagents outside the chamber. The methods used for assaying the enzymes of arginine biosynthesis have been described[10]. Formate dehydrogenase was determined according to Jones and Stadtman[11]. Assays involving extracts of *Mb. thermoautotrophicum* were performed at 55 °C; Extracts from the other organisms were incubated at 37 °C. One unit (U) of enzyme is defined as that quantity of enzyme which catalyzes the formation of 1 nmol of product per min under standard assay conditions.

Results and discussion. The data presented in the table show that all of the 3 methanogens examined possess ornithine acetyltransferase activity and an *N*-acetylglutamate 5-phospho-

Specific activities (U/mg of protein) of some arginine biosynthetic enzymes in methanogenic bacteria

Organism	Step in arginine biosynthesis*								
	Enzyme 2			Enzyme 5		Enzyme 5′		Enzyme 6	
	$-O_2$**	$+O_2$	$+O_2$ + *L*-arginine (1 mM)	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$
Methanococcus vannielii	2.6	2.9	0.4	5.7	5.1	4.1	4.5	275	290
Methanobrevibacter arboriphilus	6.9	7.4	1.2	< 1	< 1	1.5	1.5	310	326
Methanobacterium thermoautotrophicum	11.2	11.4	0.8	2.4	2.7	1.4	1.4	420	450
Pseudomonas aeruginosa	n.d.***	40	< 0.25	n.d.	3.5	n.d.	18	n.d.	150

* The trivial and systematic of the arginine biosynthetic enzymes investigated are:
enzyme 2: *N*-acetylglutamate 5-phosphotransferase (ATP: *N*-acetyl-L-glutamate 5-phosphotransferase; EC 2.7.2.8.);
enzyme 5: acetylornithinase (N^2-acetyl-*L*-ornithine amidohydrolase; EC 3.5.1.16);
enzyme 5′: ornithine acetyltransferase (N^2-acetyl-*L*-ornithine: *L*-glutamate *N*-acetyltransferase; EC 2.3.1.35);
enzyme 6: ornithine carbamoyltransferase (carbamoylphosphate: *L*-ornithine carbamoyltransferase; EC 2.1.3.3).

**The heading '$-O_2$' indicates that extract preparation and enzyme assay were performed under anaerobic conditions as described in Materials and methods. '$+O_2$' means that the anaerobically prepared extract was assayed under aerobic conditions;

***not determined.

transferase which is inhibited by arginine. Feedback inhibition of this enzyme, which catalyzes the second biosynthetic step, has been observed in all microorganisms with the ornithine acetyltransferase modification of arginine biosynthesis but not in others[3]. Our data suggest that the methanogenic bacteria examined utilize the energetically more economical version of arginine biosynthesis which involves cycling of the acetyl group between ornithine and glutamate. The ornithine acetyltransferase pathway might thus be the more ancient one of the two modifications of arginine biosynthesis observed among microorganisms. None of the 4 arginine biosynthetic enzymes tested was sensitive to oxygen. The fact that formate dehydrogenase of *Methanococcus vannielii*, an enzyme that is rapidly destroyed by oxygen[11], was measurable in anaerobically prepared extracts in the oxygen-free atmosphere of the anaerobe chamber, but not under aerobic conditions, shows that the precautions to exclude oxygen were stringent enough to detect a possible oxygen sensitivity of the arginine enzymes from methanogens. We therefore conclude that the failure (data not shown) to detect *N*-acetylglutamate synthase, the first enzyme of arginine biosynthesis, in extracts of the 3 methanogenic bacteria was not due to the destruction of its catalytic activity by oxygen.

The specific activity levels of the arginine enzymes in the methanogens listed in the table were compared with the enzyme levels in *Pseudomonas aeruginosa* PAO1, a bacterium with the ornithine acetyltransferase modification of the pathway[9]. While the levels of *N*-acetylglutamate 5-phosphotransferase and of ornithine acetylfransferase were consistently low in the methanogens, the level of ornithine carbamoyltransferase was elevated as compared to *P. aeruginosa*. Ornithine carbamoyltransferase activity in cell extracts of *Methanococcus vannielii* was measured as a function of pH. The pH curve (data not shown) had an optimum at 8.4 and its shape suggested that a single enzyme was present. This is in contrast to the situation in *P. aeruginosa* where a mixture of anabolic and catabolic ornithine carbamoyltransferases results in 2 pH optima[12]. In conclusion, 4 out of 5 arginine biosynthetic enzymes tested were readily detectable at uniform activity levels in methanogenic bacteria from 3 different genera[13]. Arginine biosynthesis thus lends itself to studies on the largely unknown amino acid metabolism of methanogens.

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