Testing the “methanochondrion concept”: are nucleotides transported across internal membranes in *Methanobacterium thermoautotrophicum*?

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Abstract. In order to test the “Methanochondrion concept”, uptake of adenine nucleotides in various membrane preparations of *Methanobacterium thermoautotrophicum* was studied. The uptake showed properties which in general are interpreted as indicative of a transport mechanism: (i) kinetics in the time range of minutes, (ii) temperature dependence, (iii) substrate specificity and (iv) failure to remove the substrate by extensive washing.

However, nucleotide transport as an interpretation of this “uptake” can definitely be excluded. Not only an exchange mechanism of the mitochondrial type, but also a general exchange or an uniport mechanism was ruled out. In contrast, the “nucleotide uptake” was shown to be actually a tight and specific binding of ADP and ATP to binding sites at the interior side of the cell membrane. This was conclusively demonstrated in protoplasts obtained from *M. thermoautotrophicum* cells. In these protoplasts which do not contain internal membranes also nucleotide binding was observed, but only after disruption of the plasma membrane by osmotic lysis, which leads to the exposure of binding sites.

Key words: *Methanobacterium thermoautotrophicum* — Nucleotide transport — Nucleotide binding — Protoplasts — Membrane vesicles — “Methanochondrion concept”

Materials and methods

The source of chemicals were the following: Nucleotides and carboxyatractylate, Boehringer (Mannheim, FRG); *14C*-labelled nucleotides and *3H*-adenine, Amersham Buchler (Braunschweig, FRG); Brij 56, Sigma Chemicals (St. Louis, MO, USA); gases and gas mixtures, Messer Griesheim (Düsseldorf, FRG); bongkrekik acid was a gift from Prof. W. Berends, Delft; all other chemicals were of analytical grade. Membrane filters (type MF) were obtained from Millipore Corp. (Neu-Isenburg, FRG), *Methanobacterium thermoautotrophicum* strain Marburg (DSM 2133) and strain ΔH (DSM 1053), *Methanosarcina barkeri* (DSM 804) and *Methanobacterium wolfei* (DSM 2970) were from the Deutsche Sammlung von Mikroorganismen (Göttingen).

Preparation of cell suspensions

*M. thermoautotrophicum* was cultured in a 500 ml glass fermenter containing 250 ml mineral salt medium on H2 and CO2 as sole carbon and energy sources, and H2S as sulfur source as described by Schönheit et al. (1980). The exponentially growing culture (at a cell concentration of 1 g
dry weight/l) was cooled down to 0°C while still gassed with the H₂, CO₂, H₂S gas mixture and the cells were anaerobically harvested and stored under a 80% H₂/20% CO₂ gas phase on ice until use (= sedimented cells).

**Preparation of membrane vesicles**

(a) Frozen/thawed cells: Sedimented cells were frozen twice at -20°C and slowly thawed at about 0—10°C. The cells were washed twice in 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 6.5, 20 mM NaCl, 1 mM MgCl₂.

(b) French press vesicles were prepared as described by Doddema et al. (1978).

(c) Sonicated vesicles: Cells were suspended at about 10—20 mg protein/ml in buffer pH 6.5 (see above). The suspension (1 ml) was sonicated in a Branson sonifier with microtip in pulse mode (20% duty, output energy 4) for 4—5 min. Undisrupted cells were removed by low speed centrifugation (10 min at 10,000 x g), vesicles were sedimented by high speed centrifugation (30 min at 175,000 x g).

**Preparation of protoplasts**

Protoplasts of *M. thermoautotrophicum* were prepared in a high osmolar buffer using an autolysate of *Methanobacterium wolfei*.

The autolysate was obtained after growth of *M. wolfei* on H₂ and CO₂ at 60°C as described by König et al. (1985). Cells of a 10 l culture were harvested in the late log phase (OD₅₇₈ = 2.5) by centrifugation. The pellet (50 g wet weight = 10 g dry weight) was suspended in 200 ml of 50 mM phosphate buffer (pH 7.0) containing 5 mM dithiothreitol and incubated under N₂ for 12 h at 60°C during which time complete lysis of cells occurred. After addition of 10 mg of DNAse, the “lysate” was centrifuged at 17,000 x g for 20 min at 4°C. The supernatant (*M. wolfei*-autolysate) was stored under 12% H₂/20% CO₂ gas phase on ice until use.

Cells of *M. thermoautotrophicum* which were used for protoplast formation were anaerobically washed and suspended in a 50 mM potassium phosphate buffer, pH 7, containing 1 M sucrose, 5 mM each of MgCl₂ and dithiothreitol and 20 μM resazurin (“phosphatesucrese-buffer”). The cell suspension (approximately 30 mg dry weight; 0.5 mg/ml) was then incubated at 60°C (gas phase: 80% H₂/20% CO₂) in the presence of 1 ml *M. wolfei*-autolysate until protoplast formation was complete (40—60 min). The protoplasts were anaerobically centrifuged at 17,000 x g for 15 min, washed once and were finally suspended in phosphate-sucrose buffer (at 10—20 mg protoplast-protein/ml) and stored under 80% H₂/20% CO₂ on ice until use.

**Measurement of nucleotide uptake**

Uptake of labelled nucleotides was measured by two different methods. Since frozen/thawed cells can easily be sedimented, uptake kinetics was analyzed by centrifugation. The uptake reaction was initiated by adding labelled substrate. After the indicated times, 50 μl of the incubation was diluted 20-fold with cold buffer (0°C) and immediately centrifuged for 1 min at 10,000 x g. The supernatant was carefully removed and the sediments were dissolved in 500 μl of 1% Brij 56 solution and subsequently counted in a liquid scintillation counter. The blank value was determined by adding unlabelled nucleotides in at least 200-fold excess before the addition of the 14C-labelled substrate. In order to make sure that no bound ligands were lost during dilution and centrifugation these results were confirmed by experiments using the silicone oil centrifugation (Heldt and Klingenberg 1967). No significant difference between the two methods could be detected.

Uptake kinetics in French press vesicles and in sonicated vesicles was analyzed by filtration through membrane filters with 0.65 μm (French press vesicles) and 0.45 μm (sonicated vesicles) pore width. After dilution in cold buffer (see above) the suspensions were filtered and the membrane filters were washed once with 1 ml of cold buffer. Blank values were obtained as described above. For counting, the filters were dried and then directly measured in scintillation fluid.

The distribution of 14C-label originating from the added 14C-nucleotide between ATP, ADP, AMP, adenosine and adenine was analyzed by ion exchange chromatography (Klingenberg et al. 1970).

Protein concentration was determined by the method of Lowry in the presence of 1% sodium lauryl sulfate (Helenius and Simons 1972) with bovine serum albumine as standard.

**Results**

Various vesicle preparations of *Methanobacterium thermoautotrophicum* show the phenomenon of adenine nucleotide uptake when incubated with labelled nucleotides. The uptake process can be resolved kinetically (Fig. 1). At room temperature it took several minutes until saturation is reached. Although uptake of labelled ATP or ADP was not observed in intact cells (Fig. 1, line A), it became detectable once the cell membrane has been opened by a freeze/thaw procedure (line B), by French press technique (line C), or by sonication (line D). The technique of freezing and thawing was used in order to open the external membranes and to render possible the access of nucleotides to the assumed intracellular vesicles. Cells from *M. thermoautotrophicum* (Marburg strain) were used under aerobic conditions in the