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AN ADENINE NUCLEOTIDE TRANSLOCASE IN THE PROCARYOTE *METHANOBACTERIUM THERMOAUTOTROPHICUM*

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SUMMARY

ATP synthesis, ATP hydrolysis and ADP uptake by membrane vesicles of *Methanobacterium thermoautotrophicum* are inhibited by inhibitors of mitochondrial ADP/ATP translocases. Atractyloside binds to one of the membrane proteins. These data demonstrate the presence of an eucaryotic type of ADP/ATP translocase in a procaryotic microorganism and stress the unique position of methanogenic bacteria in evolution.

INTRODUCTION

Recently it was shown that membrane vesicles of *Methanobacterium thermoautotrophicum*, a strictly anaerobic thermophilic bacterium, synthesize ATP from external ADP; this synthesis was sensitive to a series of mitochondrial ADP/ATP translocase inhibitors (1).This strongly suggested the presence of an ADP/ATP translocase with eucaryotic properties in this methanogenic bacterium. To date procaryotic ADP/ATP translocases have only been described in *Rickettsia prowazeki* (2) and *Rhodopseudomonas capsulata* (3). In *R. prowazeki* the ADP/ATP translocase is insensitive to inhibition by atractyloside or bongkrekic acid (2); no conclusive data are available yet for the enzyme of *R. capsulata* (3).

Methanogenic bacteria are thought to belong to the archaebacteria (4) and they may be phylogenetically different from both eubacteria and eucaryotes. The presence of an eucaryotic

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enzyme hitherto unknown to occur in procaryotes would support this concept. In this paper conclusive data are presented to show that *M. thermoautotrophicum* possesses an eucaryotic type ADP/ATP translocase.

MATERIALS AND METHODS

Growth of *M. thermoautotrophicum* strain ΔH (5), preparation of membrane vesicles (1), ATP hydrolysis (5) and ATP synthesis (1) were described previously. In the ATP hydrolysis and synthesis assays $MgCl_2$ was now replaced by $MgSO_4$. [^{14}C] ADP uptake was measured aerobically by forward exchange. Membranes (6 mg protein/ml) were incubated in 0.1 M bicine buffer, pH 8.0, with 1.25 mM $MgSO_4$ and 7 μM [^{14}C] ADP (15 Ci/mol) with or without 20 μM (carboxy) atractyloside for 15 min at room temperature. Membranes were then centrifuged through silicone oil in a Beckman Airfuge (6). After centrifugation the centrifuge tubes were frozen in liquid N_2 and cut through the silicone oil layer, and top and bottom halves were counted separately. A control run was done with 0.9 mM [^{14}C] sucrose (600 Ci/mol). Anaerobic [^{14}C] ADP uptake was measured by flow dialysis (7). Provisions were made to maintain complete anaerobiosis by flushing with H_2. Anaerobiosis was checked with the redox indicator Janus Green (0.0001%) in the flow buffer (0.1 M bicine, pH 8.0, 0.5 mM cysteine). To the ATP synthesis buffer (1) in the top half of the cell 5 mM phenazine ethosulfate or 40 μM B_o - an electron acceptor isolated from *M. thermoautotrophicum* (J. Keltjens, unpublished results) - were added to create the proper conditions for oxidative phosphorylation. Membranes (2.5 mg protein) were added to 1 ml reaction mixture, the volume of the bottom half of the cell was 1 ml, and the flow rate of the flow buffer was 1 ml/min. [^{14}C] ADP was added to a final concentration of 10 μM (20 Ci/mol). At this time the fraction collector was started. After 20 fractions had been collected Triton X-100 and EDTA were added to the reaction mixture to final concentrations of 2.5% and 10 mM, respectively. The dialysis membrane had to be treated with $ZnCl_2$ (8) and be saturated with ADP before use. Untreated dialysis membranes gave erratic results.

Anaerobic binding of [^{3}H] atractyloside (13.5 Ci/mol) (kindly supplied by Prof. P.V. Vignais) to membranes was measured by incubation of 50 μl membranes (10 mg protein/ml) with 1 μM [^{3}H] atractyloside at 65°C under H_2 in 0.1 M bicine buffer, pH 8.0, for 15 min, followed by the addition of Triton X-100 (2.5%) (9) and anaerobic separation by disc gel electrophoresis. Gels were stained for protein, or cut in slices (1 mm) which were extracted with 0.5 ml 10% Triton X-100 at 60°C for 24 h, and tested for radioactivity.

RESULTS AND DISCUSSION

Forward exchange ADP uptake was inhibited about 20% by 20 μM atractyloside (Table 1). About 200 pmol/mg protein of

Table 1. Uptake of [^{14}C] ADP.

Incubation mixture	% Label in membranes
[^{14}C] sucrose	5 ± 2
[^{14}C] ADP	21 ± 2
[^{14}C] ADP + 20 μM atractyloside	16 ± 2

Incubation as given in the Methods section. Values are the means of triplicate experiments.

[^{14}C] ADP were associated with the membrane pellet. ATP synthesis is inhibited between 20 and 80% (1) by 20 μM atractyloside. The low inhibition found in ADP uptake may be due to the aerobic conditions; also respiratory priming may be necessary for higher inhibition as in some plant mitochondria (10). To test this hypothesis [^{14}C] ADP uptake was measured by anaerobic flow dialysis under conditions favouring oxidative phosphorylation (Fig. 1). About 300 pmol ADP/mg protein were taken up, slightly more than in the aerobic uptake experiment, but uptake was completely inhibited by 20 μM atractyloside.

In the [^{3}H] atractyloside binding experiment followed by separation of proteins on a polyacrylamide gel, radioactivity was found in one zone, which corresponded to a protein band. Obviously atractyloside binds to one of the membrane proteins.

ATP hydrolysis in the presence of either atractyloside or carboxyatractyloside is also inhibited (Table 2). Concentrations of 1 to 25 μM inhibitor reduce the ATP hydrolysis rate about 25%. Supposing complete inhibition of the adenine nucleotide translocase, one may conclude that about 75% of the ATP-ase is associated with inside-out membranes.

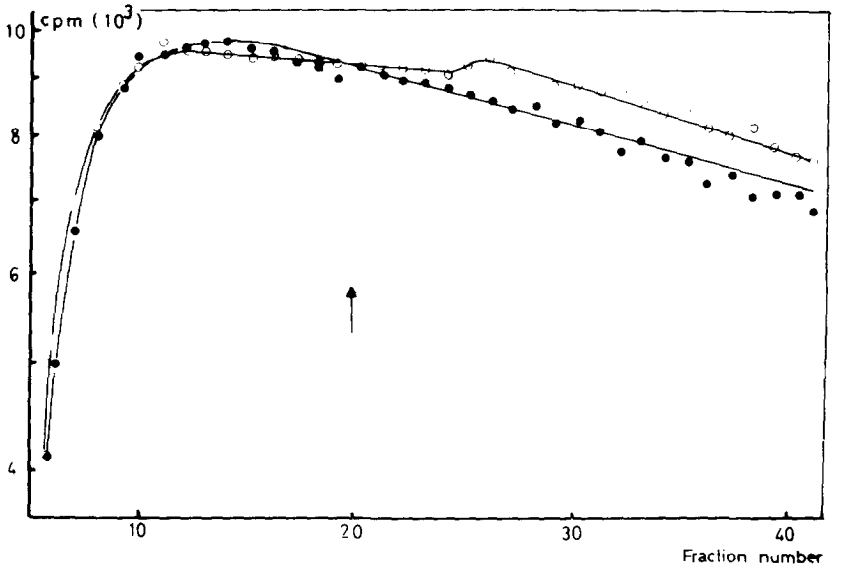

Fig. 1. Uptake of [^{14}C] ADP by membrane vesicles of *M. thermoautotrophicum* measured by flow dialysis. Incubation conditions as described in the Methods section. The delay between the flow cell and the fraction collector was about 6 fractions. The arrow indicated the addition of Triton X-100 (2.5%) and EDTA (10 mM). o ADP uptake without inhibitor, • ADP uptake with 20 μM atractyloside added. The results shown are from a typical run.

Table 2. Inhibition of ATP hydrolysis.

Additions to control	ATP hydrolysis as % of control
none	100
atractyloside	80
carboxy-atractyloside	70
agaric acid	200
atractyloside + 1% toluene	130

Inhibitors added to final concentration of 20 μM. Incubation as given in Methods section. Experiments were performed at least in quintuplicate. The s.d. is 5%.

The effect of agaric acid - a strong enhancement of ATP hydrolysis - was only apparent after an incubation of 2 min. In assays for ATP synthesis the presence of agaric acid results in an inhibition which is apparent by a slower rate of ATP synthesis during the first two minutes followed by rapid ATP hydrolysis. A possible uncoupling action of this lipophilic weak acid cannot yet be excluded. The precise nature of the effect of agaric acid needs further study.

The inhibition of ATP hydrolysis by the ADP/ATP translocase inhibitors atractyloside and carboxyatractyloside could be relieved by making the membranes permeable with 1% toluene or bee venom (25 μg/ml).

The substrate for the ADP/ATP translocase is free ADP rather than the Mg-ADP complex (11). Lowering the Mg^{2+} concentration from 1.25 mM to 0.25 mM enhanced ATP synthesis by a factor two. This is in agreement with the substrate specificity observed for the mitochondrial ADP/ATP translocase.

The inhibition of ATP synthesis, ATP hydrolysis and ADP uptake by the mitochondrial ADP/ATP translocase inhibitors, and the binding of atractyloside to a membrane protein would seem to place the presence of an eucaryotic type of ADP/ATP translocase in the procaryote *M. thermoautotrophicum* beyond reasonable doubt. ATP synthesis is localised on the internal membranes of this organism (1), but the exact function of the translocase will remain obscure until the orientation of the membrane-bound hydrogenase and ATP-synthetase has been fully clarified. However, the presence of the ADP/ATP translocase completes the picture of a procaryotic organelle which resembles the mitochondrion in its function. These data indicate that the methanogenic bacteria may indeed be phylogenetically distinct from both the eubacteria and the eucaryotes.

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