Sodium-dependent isoleucine transport in the methanogenic archaebacterium *Methanococcus voltae*

Ken F. Jarrell, Sharon E. Bird and G. Dennis Sprott

Division of Biological Sciences, National Research Council of Canada, Ottawa K1A OR6, Canada

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Methanococcus voltae possesses a Na^+-dependent transport system for isoleucine which requires for optimum rates a CO_2/H_2 atmosphere. The K_m for the system is 4.5 μM with a V_{max} of 1.5 nmol · min^{-1} · mg dry wt^{-1}. Approximately 75% of the label can be released from the cell pool following short-term experiments with gradients of isoleucine reaching 100 (in/out). Transport is inhibited by ionophores and *N*-ethyl maleimide. Only valine and leucine effectively compete with isoleucine for transport.

Sodium-dependent transport *Isoleucine* Methanococcus voltae

1. INTRODUCTION

There are only 3 reports of transport systems in methanogenic bacteria. These are coenzyme M transport in *Methanobrevibacter ruminantium* [1], Ni^{2+} transport in *Methanobacterium bryantii* [2] and an ADP/ATP translocase in membrane vesicles of *Methanobacterium thermoautotrophicum* [3]. Recently, however, it was reported [4] that *Methanococcus voltae* required isoleucine and leucine for growth in a defined medium, a finding which suggested the presence of a transport system for these amino acids. Here we document the presence of a high affinity Na^+-dependent isoleucine transport system in *M. voltae*.

2. MATERIALS AND METHODS

2.1. *Organism and medium*

Methanococcus voltae, obtained from Dr Len Hook, Ohio State University, was grown with gentle shaking in 100 ml vols of Balch medium III [5] using modified 1-l Wheaton bottles [1]. The gas atmosphere was CO_2/H_2 (1:4, v/v).

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2.2. *Transport assay*

Cells (10 ml) were centrifuged anaerobically [6] at 5000 × *g* for 10 min and unless otherwise stated washed once in anaerobic H_2S-reduced 100 mM Hepes buffer (pH 6.5), containing 0.4 M sucrose, 10 mM KCl, 10 mM $MgCl_2$ and 50 mM NaCl. The washed cell pellet was resuspended in the same buffer solution (5 ml) and transferred to a 60-ml serum bottle under CO_2/H_2 (1:4, v/v) at 35°C with gentle shaking. Isoleucine (^3H or ^{14}C, New England Nuclear of Canada, Lachine, Quebec) was added to a final concentration of 50 μM and samples removed at timed intervals, filtered and washed. The filter system consisted of an HA filter (0.45 μm, Millipore) overlayered with a Whatman no. 1 prefilter to increase the rate of filtration. Examination of the transport system with various gas atmospheres and in the presence of inhibitors and competitors was as in [2]. The internal volume of *M. voltae* was 1.37 μl/mg dry w of cells, determined as the difference between penetrations of urea and sucrose [7,8].

2.3. *Metabolism of isoleucine*

Cells were incubated with labeled isoleucine and samples were removed and filtered at timed intervals. The filters were washed with either the washing and resuspending buffer or with 5% (w/v) trichloroacetic acid. The difference in radioactivity

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between the total uptake and the trichloroacetic acid insoluble fraction was taken as the free isoleucine pool [9,10]. As a further test of metabolism, a cell suspension was incubated with [^{14}C]isoleucine for 10 min, air introduced to terminate uptake, and centrifuged. The pellet was washed and the cells lysed in a minimum volume of water. After boiling for 5 min and centrifuging, the supernatant was spotted on precoated cellulose thin-layer chromatography plates and developed in either propan-2-ol: formic acid:water (40:5:10, by vol. [11]) or *n*-butanol:acetic acid:water (25:4:10, by vol.). Spots corresponding to isoleucine were removed, the radioactivity determined and compared to the total amount spotted.

3. RESULTS

Since *M. voltae* was grown in the complex Balch III medium [5], the cells were washed and resuspended in a buffer solution before use in transport studies. *M. voltae* are very fragile [4] but cells undergoing this treatment retained their normal phase dark appearance. Furthermore, the buffer-treated cells produced CH_4 at >80% of the rate observed with comparable cells washed and resuspended in Balch medium III (not shown).

M. voltae transported isoleucine by a Na^+-dependent mechanism (fig.1). The small amount of uptake (30 pmol · min^{-1} · mg dry wt) occurring in the absence of added Na^+ probably resulted from contaminating amounts of Na^+ in the transport solution. The rate of uptake increased dramatically as Na^+ was added to the system with a broad optimum of 50–100 mM observed. Inhibition of transport occurred at higher Na^+ concentrations where a plasmolysis of the cells was observed in the transport solution. Although KCl at the same concentration could not substitute for NaCl, LiCl had some capacity to substitute giving approx. 23% of the transport rate observed with NaCl (fig.2).

Isoleucine transport specifically required a CO_2/H_2 atmosphere. Transport under an atmosphere of N_2, CO_2/N_2 or air was 10% of the rate observed with CO_2/H_2 (fig.3). Transport was optimal at 30–40°C and was routinely tested at 35°C.

Under optimal conditions with 50 mM NaCl pre-

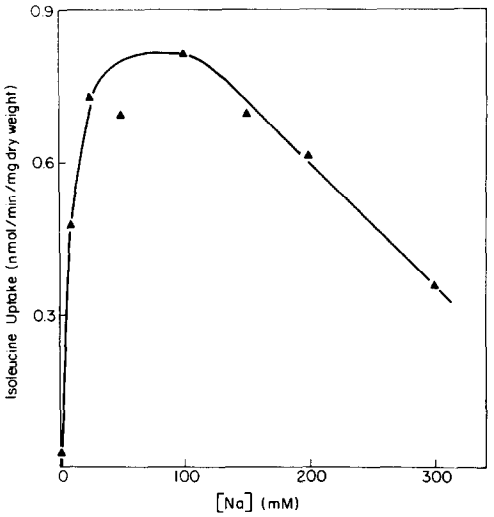

Fig. 1. The sodium dependence of isoleucine transport in *M. voltae*. Cells were washed and resuspended in 100 mM Hepes buffer (pH 6.5) containing 0.4 M sucrose, 10 mM KCl, 10 mM $MgCl_2$ and the indicated concentration of NaCl. Isoleucine was added to a final concentration of 50 μM.

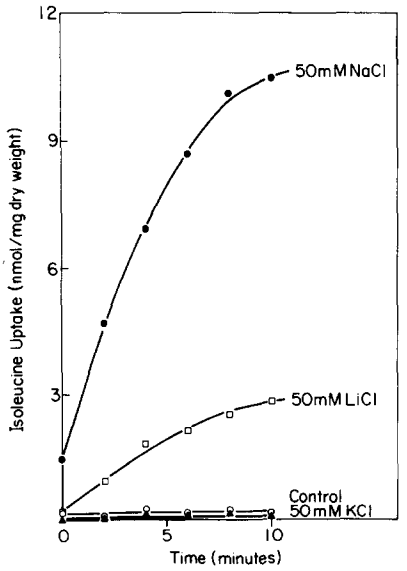

Fig. 2. The cation specificity of isoleucine transport in *M. voltae*. Cells were washed and resuspended in 100 mM Hepes buffer (pH 6.5) containing 0.4 M sucrose, 10 mM KCl, 10 mM $MgCl_2$ plus 50 mM of the indicated chloride salt. (●) NaCl; (□) LiCl; (▲) KCl; (○) no addition. The final isoleucine concentration was 50 μM.

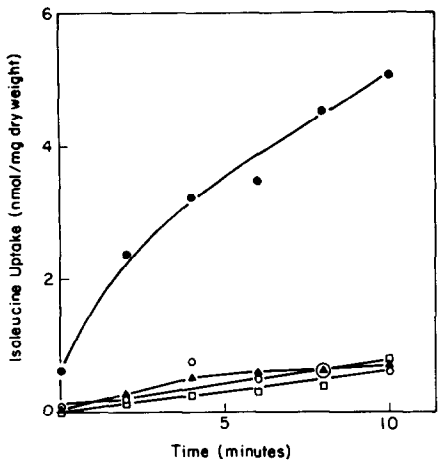

Fig. 3. Effect of headspace gas on isoleucine transport in *M. voltae*. Cells were washed and resuspended in 100 mM Hepes buffer (pH 6.5) containing 0.4 M sucrose, 10 mM KCl, 10 mM $MgCl_2$ and 50 mM NaCl and transferred to 60-ml serum bottles under the indicated headspace for 30–45 min before the addition of 50 μM isoleucine. The pH values of the cell suspensions at the end of the experiment were: (●) CO_2/H_2, pH 6.23; (○) CO_2/N_2, pH 6.25; (▲) N_2, pH 6.49; (□) air, pH 6.50.

sent, the apparent K_m was 4.5 μM with a V_{max} of 1.5 nmol · min^{-1} · mg dry wt^{-1}.

The presence of a number of ionophores and inhibitors greatly decreased the rate of isoleucine transport (table 1). The inhibition by NEM, a sulphydryl reagent, suggested that transport was carrier-mediated. This was further substantiated by competition experiments with various amino acids. The branched-chain amino acids valine and leucine, which are often transported by the same system as isoleucine [9,12] were very strong inhibitors of isoleucine transport; other amino acids tested were much less effective (table 2).

The ability of *M. voltae* to concentrate isoleucine was examined. The difference in radioactivity between the total uptake (buffer-washed cells) and that incorporated trichloroacetic acid-washed cells) was taken as the free isoleucine pool. Approximately 20–25% of the total label accumulated was found to be trichloroacetic acid-precipitable. This value was similar regardless of whether the incubation mixture contained 1 mM propionate or 1 mM 2-methylbutyrate, compounds which can be used to synthesize isoleucine by a variety of methanogens [13]. Thus catabolism of isoleucine following transport is likely to be minimal since biosynthesis of isoleucine from the fatty acids should have diluted the labeled-isoleucine pool and resulted in higher recoveries of [^{3}H]isoleucine (fig.4). Thin-layer chromatography of the pool material confirmed that the majority of the label comigrated with authentic isoleucine in two different solvent systems. Based on the finding that approximately 25% of the total accumulated label is incorporated and 75% is free isoleucine, concentration gradients of >100 (in/out) can be achieved.

TABLE 1

Effect of ionophores and inhibitors on isoleucine transport in *M. voltae*

Treatment	Isoleucine transport (%)[a]
Ethanol (control)	100
Heat treatment (95°C/10 min)	0.6
TCS (20 μM)[b]	0.4
CCCP (20 μM)[b]	14.8
Monensin (20 μM)	10.9
Nigericin (20 μM)	10.2
TPP^+ (1 mM)[b]	32.9
N-Ethyl maleimide (1 mM)	3.5

[a]Control uptake was 939 pmol isoleucine · min^{-1} · mg dry wt^{-1}

[b]TCS, 3,3′,4′,5-tetrachlorosalicylanilide; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TPP^+, tetraphenylphosphonium cation

TABLE 2

Effect of various amino acids on isoleucine transport by *M. voltae*

Competing amino acid[a]	Initital rate (%)[b]
None	100
Isoleucine	3.2
Leucine	9.9
Valine	12.7
Alanine	101.0
Phenylalanine	88.6
Methionine	60.0
Glycine	85.5

[a]Each competing amino acid was added simultaneously with [^{3}H]isoleucine. The final concentration of the cold amino acids was 1 mM while [^{3}H]isoleucine was 50 μM

[b]100% = 1.2 nmol · min^{-1} · mg dry wt^{-1}

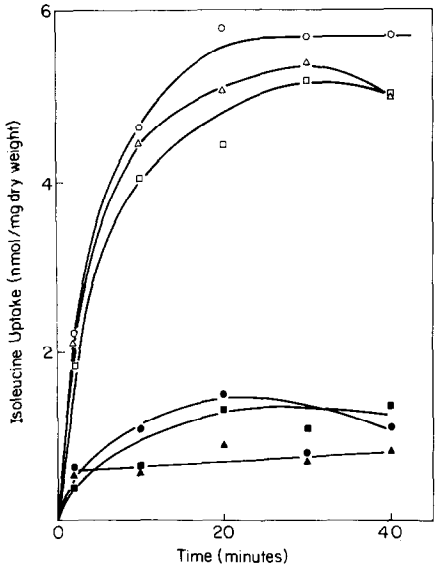

Fig. 4. Time-course of isoleucine distribution in *M. voltae*. Isoleucine (50 μM) was added to cells suspended in 100 mM Hepes (pH 6.5) containing 0.4 M sucrose, 10 mM KCl, 10 mM $MgCl_2$ and 50 mM NaCl. At the indicated times samples were removed, filtered, and washed with either the resuspending buffer (open symbols) or with 5% (w/v) trichloroacetic acid (solid symbols). Cells were preincubated for 15 min with 1 mM sodium propionate (□,■), 1 mM 2-methylbutyrate (△,▲) or no addition (○,●).

4. DISCUSSION

This report documents the first carrier-mediated amino acid transport system and the first instance of Na^+-dependent transport in a methanogenic bacterium. The system is a high affinity, active transport system which is optimal only under a CO_2/H_2 atmosphere in the presence of 50–100 mM NaCl. Transport is inhibited by ionophores and concentration gradients of >100 can be achieved. The transport system is apparently a branched-chain amino acid-specific system like the LIV systems of *E. coli* and *Pseudomonas aeruginosa* [9,12].

In [14,15] it was shown that several methanogens require Na^+ for growth and CH_4 production. *M. voltae* was not included in these studies, but authors in [4] demonstrated a Na^+ requirement for growth and we have observed that Na^+ stimulates CH_4 synthesis in *M. voltae* (not shown).

The precise role which Na^+ may play in energy conservation of transport in methanogenic bacteria is unknown. The Na^+ dependence may be the result of Na^+ effects on the binding affinity of a transport protein for the substrate or to effects of Na^+ on the driving force for isoleucine transport [16]. The energetics of transport can readily be explained by invoking a Na^+-amino acid symport model. When metabolizing CO_2/H_2 in growth media of pH 6.5–6.8, the methanogens examined thus far lack significant transmembrane pH gradients and $\Delta\psi$ (negative inside) is the predominant component of the proton motive force [6,8,17]. Assuming a Na^+ symport model, this suggests an electrogenic influx of Na^+ with isoleucine via a positively-charged carrier complex. Na^+-dependent cotransport systems driven by $\Delta\psi$ have been found in *Chromatium vinosum* [18] and other bacteria [16]. Support for the model was obtained by showing transport inhibition by compounds expected to dissipate $\Delta\psi$ (protonophores or TPP^+). Studies are in progress to measure the effects of the ionophores on the proton motive force in *M. voltae*.

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