

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

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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 $4.5 \mu\text{M}$ with a V_{\max} of $1.5 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg dry wt}^{-1}$. 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.

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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).

2.2. Transport assay

Cells (10 ml) were centrifuged anaerobically [6] at $5000 \times g$ 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 $50 \mu\text{M}$ and samples removed at timed intervals, filtered and washed. The filter system consisted of an HA filter ($0.45 \mu\text{m}$, Millipore) overlaid 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 $1.37 \mu\text{l}/\text{mg dry w}$ 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 ($30 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg dry wt}$) 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-

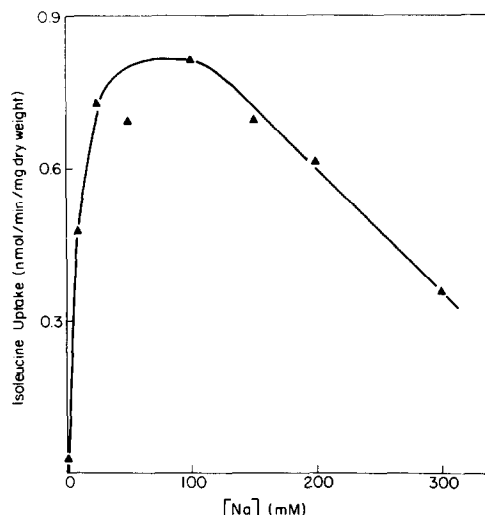


Fig. 1. The sodium dependence of isoleucine transport in *M. voltae*. Cells were washed and resuspended in 100 mM Hepes buffer (pH 6.5) containing 0.4 M sucrose, 10 mM KCl, 10 mM MgCl_2 and the indicated concentration of NaCl. Isoleucine was added to a final concentration of 50 μM .

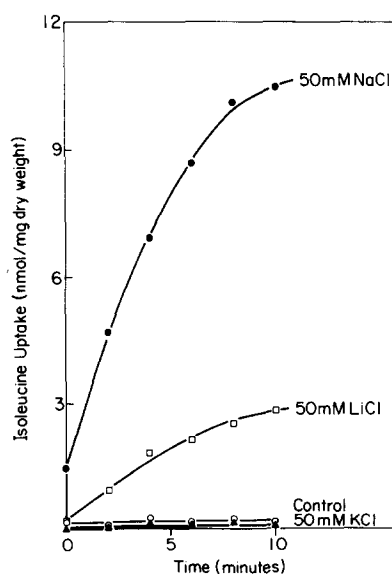


Fig. 2. The cation specificity of isoleucine transport in *M. voltae*. Cells were washed and resuspended in 100 mM Hepes buffer (pH 6.5) containing 0.4 M sucrose, 10 mM KCl, 10 mM MgCl_2 plus 50 mM of the indicated chloride salt. (●) NaCl; (□) LiCl; (▲) KCl; (○) no addition. The final isoleucine concentration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

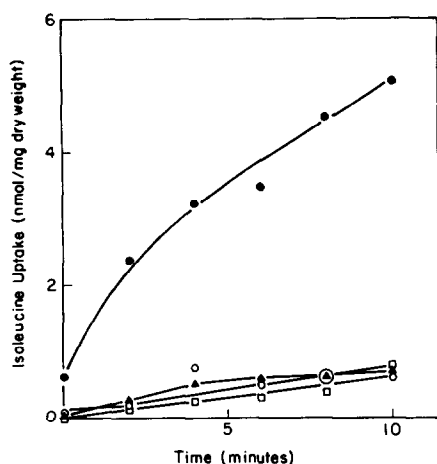


Fig. 3. Effect of headspace gas on isoleucine transport in *M. voltae*. Cells were washed and resuspended in 100 mM Hepes buffer (pH 6.5) containing 0.4 M sucrose, 10 mM KCl, 10 mM MgCl₂ and 50 mM NaCl and transferred to 60-ml serum bottles under the indicated headspace for 30–45 min before the addition of 50 μ M isoleucine. The pH values of the cell suspensions at the end of the experiment were: (●) CO₂/H₂, pH 6.23; (○) CO₂/N₂, pH 6.25; (▲) N₂, pH 6.49; (□) air, pH 6.50.

sent, the apparent K_m was 4.5 μ M with a V_{max} of 1.5 nmol \cdot min⁻¹ \cdot 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

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 μ M) ^b	0.4
CCCP (20 μ M) ^b	14.8
Monensin (20 μ M)	10.9
Nigericin (20 μ M)	10.2
TPP ⁺ (1 mM) ^b	32.9
N-Ethyl maleimide (1 mM)	3.5

^aControl uptake was 939 pmol isoleucine \cdot min⁻¹ \cdot mg dry wt⁻¹

^bTCS, 3,3',4',5-tetrachlorosalicylanilide; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TPP⁺, tetraphenylphosphonium cation

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 [³H]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 2

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

Competing amino acid ^a	Initial 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

^aEach competing amino acid was added simultaneously with [³H]isoleucine. The final concentration of the cold amino acids was 1 mM while [³H]isoleucine was 50 μ M

^b100% = 1.2 nmol \cdot min⁻¹ \cdot mg dry wt⁻¹

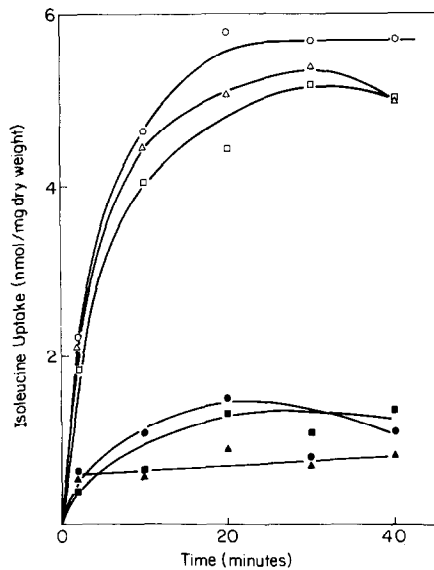


Fig. 4. Time-course of isoleucine distribution in *M. voltae*. Isoleucine (50 μ M) was added to cells suspended in 100 mM Hepes (pH 6.5) containing 0.4 M sucrose, 10 mM KCl, 10 mM $MgCl_2$ and 50 mM NaCl. At the indicated times samples were removed, filtered, and washed with either the resuspending buffer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

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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