Amino acid transport in membrane vesicles of *Clostridium thermoautotrophicum*

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1. SUMMARY

A proton motive force (Δp) generated by oxidation of CO in membrane vesicles of *Clostridium thermoautotrophicum* drove active transport of L-alanine, glycine and L-serine. The maximum rate (V\text{max}) for L-alanine transport was 12 × higher at 50°C than at 25°C. The apparent transport constant (Kt) for L-alanine uptake was 30–40 μM and independent of the temperature. Glycine was a substrate for the L-alanine transport system as demonstrated by the competitive inhibition of L-alanine uptake by glycine (K\text{in} = 6 μM), by the kinetics of glycine uptake (K\text{in} = 7 μM) and by the inhibition of glycine uptake by L-alanine. The uptake kinetics of glycine was biphasic. L-Serine inhibited competitively also L-alanine and glycine transport but it was taken up by a separate transport system. The rate of amino acid transport, but not the Kt, was dependent on the value of the proton motive force.

2. INTRODUCTION

*Clostridium thermoautotrophicum*, a thermophilic homoacetogenic bacterium grows heterotrophically and autotrophically [1]. In the latter case, CO₂ and hydrogen or CO serve as sole carbon and energy sources. Under all conditions acetate is synthesized via the Wood acetyl-CoA pathway [2–4]. The reduction of CO₂ to acetate is coupled to energy generation by a membrane associated chemiosmotic process [5,6]. Membrane vesicles prepared from *C. thermoautotrophicum* by osmotic lysis contain an active electron transport chain and carbon monoxide dehydrogenase. These vesicles generate by CO oxidation a proton motive force (Δp) which drives the synthesis of ATP [7] and the active uptake of alanine [6]. In this paper we have focused on transport of glycine, L-alanine and L-serine driven by the oxidation of CO₂, and demonstrate that membrane vesicles of *C. thermoautotrophicum* offer a novel system to study amino
acid transport under anaerobic conditions without the interference of cytoplasmic activity.

3. MATERIALS AND METHODS

3.1. Bacterium and membrane vesicles preparation

*C. thermoautotrophicum* 701/5, was grown on glucose under CO₂ atmosphere at 59°C, pH 6.5 [8]. Membrane vesicles were prepared from fresh cells only as described [5,9].

3.2. Measurement of the proton motive force

The proton motive force was determined by distribution of the lipophilic cation TPP⁺ over the cytoplasmic membrane [6] and from the chemical gradient of the transport glycine or L-alanine by assuming that ∆p = ∆μ_{sub}. Calculations of the ∆p using the latter method invariably yield high values which was noted and discussed previously [6].

3.3. Transport assays

Amino acid uptake into membranes was measured under anaerobic conditions as described [6]. Carbon monoxide was the electron donor and ferricyanide (10 mM) the electron acceptor. The assays were performed in a volume of 100 μl of 100 mM potassium phosphate, pH 7.0 containing 45 μg of membrane protein. Ferricyanide was added 3 min before the addition of radioactive substrate at 25°C and 1 min before at 50°C to make sure that maximum values of the proton motive force had been reached before the start of the uptake experiment. Initial rates of amino acid transport were calculated from the period during which the uptake was linear with time. This time period ranged from 10 s (high concentrations, 50°C) to 5 min (low concentrations, 25°C). Substrate concentrations varied from 1.5 μM to 75 μM for L-alanine, from 3.5 μM to 550 μM for glycine and from 2.5 μM to 125 μM for L-serine. The rate of amino acid transport (v and V_{max}) is expressed in nmol·min⁻¹·mg⁻¹ of protein. L-alanine, glycine, L-serine, L-threonine, L-leucine, proline β-alanine or pyruvate used as inhibitors were added immediately before the addition of the radioactive amino acid at the concentration of the latter or ten-fold higher. V_{max} and Kᵢ values were determined graphically using Eadie-Hofstee plots [10].

3.4. Other assays and chemicals

Carbon monoxide dehydrogenase was assayed as described previously [11]. Protein was measured by the rose bengal dye-binding assay [12] in the presence of 0.2% Triton X-100. Bovine serum albumin was used as the standard. All radioactive chemicals were purchased from Amersham Corporation (Arlington Heights, IL).

4. RESULTS

4.1. Kinetics of L-alanine transport

The initial rates of L-alanine uptake were determined at 25°C and at 50°C using membranes that generated a ∆p of -159 mV. The maximum uptake rate (V_{max}) obtained from the Eadie-Hofstee plots (Fig. 1) was approximately 12 fold higher at 50°C (V_{max} = 23) than at 25°C (V_{max} = 2.0), while the Kᵢ values remained about the same (35 μM and 40 μM, respectively at the two temperatures). The increase in V_{max} with temperature was not due to changes in the driving force for uptake since the ∆p did not vary with the temperature [6].

The V_{max} of uptake of L-alanine was strongly dependent on the magnitude of the ∆p generated in the membrane vesicles and an exponential relationship was found (Fig. 2). The V_{max} varied from 0.3 at ∆p = -65 mV to 23 at ∆p = -160 mV. The Kᵢ (35 μM to 40 μM) for L-alanine remained much the same over this range of ∆p values. As noted earlier, the ∆p of the membrane vesicles is dependent on the activity of the CO dehydrogenase of the vesicles [6]. This activity varies between membrane preparations, depending on growth state of the bacteria when harvested and on how carefully anaerobic conditions are maintained during the preparation.

Glycine and L-serine, but not β-alanine, L-leucine, L-proline, L-threonine and pyruvate, inhibited L-alanine transport in an apparently competitive manner with apparent inhibition constants (Kᵢ) of 6 μM and 40 μM, respectively (data not shown).
4.2. Characteristics of glycine transport

Glycine was actively transported by membrane vesicles of *C. thermoautotrophicum*. As shown in Fig. 3 the kinetics of this transport was biphasic. At low concentrations of glycine (1 µM to 35 µM) the apparent $K_i$ was 7 µM: a value close to the $K_i$ of 6 µM found for glycine inhibition of L-alanine transport. Transport of glycine at these low concentrations was inhibited by L-alanine in a competitive manner, exhibiting a $K_i$ of 28 µM which is close to the $K_i$ for L-alanine transport. The $V_{max}$ for glycine transport appeared to be slightly lower than that for L-alanine transport. This is seen in Table 1, which displays data obtained from membrane vesicles generating a $\Delta \rho$ of -120 mV. The results indicate that glycine at low concentrations is transported by the same system as that of L-alanine. The biphasic nature of the Eadie-Hofstee plot (Fig. 3) indicates that at higher glycine concentrations (50 µM to 550 µM) a transport system for glycine operates that is different from that at low concentrations and which has a $K_i$ of approximately 500 µM and a $V_{max}$ 10 fold higher (Table 1). This transport system was also inhibited by L-alanine with an apparent $K_i$ of 280 µM.

4.3. Characteristics of L-serine transport

L-serine inhibited competitively both the transport of L-alanine ($K_i = 40$ µM) and that of glycine ($K_i = 30$ µM) (Table 1). This suggested that L-serine was transported by the same system as used for these other amino acids. L-Serine was indeed actively taken up by membrane vesicles of *C. thermoautotrophicum* with a $K_i$ of 60 µM and a $V_{max}$ of 1.0 (Table 1). The transport of L-serine, however, was not inhibited by L-alanine nor by...
glycine, not even at 100 fold higher concentrations. Only L-threonine appeared to be an inhibitor of L-serine transport though weakly with a \( K_i \) of approximately 500 \( \mu \)M. We feel that L-serine is a specific inhibitor of but not transported by the L-alanine/glycine transport system and that L-serine is transported by a separate system. If this system also transports L-threonine has not been investigated.

5. DISCUSSION

Transport of amino acids has not been studied extensively in clostridia or other anaerobic bacteria. This may be due to difficulties in working with strict anaerobes, but perhaps more importantly to the lack of energy generating systems in membranes of anaerobes. To overcome the latter problem proton pumps such as cytochrome oxidase, bacteriorhodopsin and reaction centers of phototrophic bacteria have been artificially introduced into membranes of \textit{Lactococcus lactis} (formerly \textit{Streptococcus cremoris}) \cite{13–15}, \textit{Clostridium acetobutylicum} \cite{16,17} and the thermophilic \textit{Clostridium fervidus} \cite{18}. As shown in this paper, membrane preparations of \textit{C. thermoautotrophicum} allow a more direct, natural and convenient way of studying substrate transport in anaerobes since they possess a membrane associated CO dehydrogenase and an anaerobic electron transport system which generate a \( \Delta \psi \) using CO as electron donor \cite{5,6}. A further advantage of the \textit{C. thermoautotrophicum} system is that it allows studies of active transport into membrane vesicles at a wider temperature range (up to 65 °C) than described before in other thermophiles \cite{18,19}.

The transport systems for L-alanine/glycine and L-serine in \textit{C. thermoautotrophicum} are comparable in substrate specificity and activity \((V_{max})\) at 25° C to the systems of \textit{Escherichia coli}, \textit{Bacillus subtilis}, and \textit{L. lactis} \cite{13,15,20,21}. The specific rates are much higher than those reported for the obligate anaerobe, \textit{C. acetobutylicum} \cite{16,17}. However, transport rates are dependent on the magnitude of the \( \Delta \psi \) as shown here and by others \cite{13,16,22} and thus are difficult to compare.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Amino acid & \( K_i \) & \( V_{max} \) & Inhibitors (\( K_i \)) & L-Ala & Gly & L-Ser & L-Thr \\
\hline
L-Ala & 35 & 4.1 & – & 6 & 40 & > 500 \\
Gly 1 & 7 & 3.5 & 28 & – & 30 & > 500 \\
Gly 2 & 500 & 30.0 & 280 & – & n.d. & n.d. \\
L-Ser & 60 & 1.0 & > 500 & > 500 & – & 500 \\
\hline
\end{tabular}
\caption{Summary of kinetic constants of transport of L-alanine, glycine and L-serine into membrane vesicles of \textit{C. thermoautotrophicum}.}
\end{table}
Biphasic kinetics was observed for glycine transport in \textit{C. thermoautotrophicum}. This phenomenon observed also in other bacteria is generally explained by the presence of more than one transport system for the particular substrate. However, for lactose permease of \textit{E. coli} \cite{22} biphasic kinetics is apparently due to two active forms of one transport system and biphasic leucine transport in \textit{L. lactis} is due to passive diffusion of the substrate through the membrane at high leucine concentrations \cite{13}. The latter is not the case of glycine transport in \textit{C. thermoautotrophicum} since it was competitively inhibited by L-alanine at high glycine concentrations demonstrating the enzymatic nature of the transport.

Interestingly, the amino acid transport systems in the thermophilic bacteria PS-3, \textit{B. stearothermophilus} and \textit{C. fervidus} appear to be Na$^+$ solute symport systems \cite{18,19,23}. In \textit{C. thermoacetietum} and \textit{C. thermoautotrophicum} we have found no indication for a role of sodium in amino acid transport. The uptake of alanine, glycine and serine in these thermophiles was not affected by the absence of presence of Na$^+$ ions (data not shown).

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