Sodium-Dependent Transport of Branched-Chain Amino Acids by a Monensin-Sensitive Ruminal Peptostreptococcus

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For many years it had been assumed that most, if not all, of the predominant bacteria had been isolated from the rumen, but these species could not account for in vivo rates of amino acid degradation (2). Recently, isolated ammonia-producing ruminal bacteria (6, 21) grew rapidly with amino acids or peptides as their sole energy source and were present in significant numbers in vivo. One of them, a peptostreptococcus, produced large amounts of branched-chain volatile fatty acids, and these acids arose from the fermentation of branched-chain amino acids (5).

Monensin is often used as a feed additive in beef cattle, and it inhibits amino acid deamination (9, 27). The reduction in wasteful amino acid fermentation is desirable, but monensin can also decrease branched-chain volatile fatty acid production (13, 17). These acids are required by ruminal cellulolytic bacteria, and a deficiency can inhibit cellulose digestion (1). Because branched-chain volatile fatty acids can have a significant effect on feed digestion (23), we decided to examine the metabolism of branched-chain amino acids by the ruminal peptostreptococcus in greater detail.

The rumen is an Na-rich environment (approximately 90 mM), and results indicated that the peptostreptococcus took up leucine, valine, and isoleucine by a common carrier which was dependent on Na. The carrier had a single site for Na (Km of 5.2 mM) and could be driven by an Na+ gradient in the absence of either a ∆ψ or Z∆pH. Growth was inhibited by monensin, an Na+-H+ antiporter, but this effect appeared to be more closely correlated with a decrease in ATP than a decrease in transport activity or proton-motive force.

MATERIALS AND METHODS

Cells. The ruminal peptostreptococcus strain was used and this bacterium has previously been described (5, 21). Cells were grown anaerobically in medium containing (per liter) 292 mg of KH2PO4, 292 mg of KH2PO4, 480 mg of Na2SO4, 480 mg of NaCl, 100 mg of MgSO4·7H2O, 64 mg of CaCl2·2H2O, 600 mg of cysteine hydrochloride, 15 g of Casamino Acids (Difco Laboratories, Detroit, Mich.), vitamins, and microminerals (7). The medium pH was 6.7, and incubation temperature was 39°C. The peptostreptococcus was also inoculated into Na-deficient medium which contained K salts instead of Na salts, 1 g of purified amino acids per liter with a composition similar to Casamino Acids as a carbon source, and 15 g of leucine per liter as an energy source. Na dependency was ascertained by supplementing this Na-deficient medium with 100 mM NaCl. Batch cultures were grown overnight to an optical density of approximately 1.0 (600 nm, 1 cm). Cells were harvested by centrifugation (11,000 × g, 5 min, 25°C) and washed twice in potassium phosphate buffer (100 mM, pH 6.5). Supernatants and some cell pellets were stored at −15°C for further analysis. Membrane vesicles were prepared as previously described (22).

Transport assays. Washed cells (or vesicles) were treated with vanilomycin (5 μM) and incubated in 100 mM potassium phosphate (pH 6.5) to load them with K. K-loaded cells were diluted 50-fold into 100 mM choline or sodium phosphate (pH 6.5) to create an artificial ∆ψ or a ∆ψ and a chemical gradient of Na+ (∆uNa+). The effect of Na was monitored by diluting K-loaded cells into choline phosphate buffer, which was supplemented with NaCl (0 to 100 mM). pH experiments were conducted with cells which were incubated in potassium phosphate (pH 4.5 to 7.5) and diluted into the same pH. An artificial Z∆pH was created by diluting K (100 mM)- and acetate (80 mM, pH 6.5)-loaded cells into potassium phosphate (100 mM, pH 6.5). Cells were sometimes treated with monensin (5 μM) during K loading and diluted into 100 mM sodium phosphate.

Transport was initiated by adding 4 μl of concentrated
cells (40 µg of protein) to 200 µl of phosphate buffer (described above). After 0 to 60 s, transport was terminated by adding 2 ml of 100 mM ice-cold LiCl or 100 mM KCl and filtering through cellulose nitrate membrane filters (0.45-µm pore size). The filters were washed once with 2 ml of 100 mM LiCl and dried for 20 min at 105°C. Filters were then counted by liquid scintillation. Competition experiments were conducted with a 100-fold excess of unlabeled amino acid.

The peptostreptococcus transported branched-chain amino acids very rapidly, and the rate of transport over 5 s was proportional to cell protein as long as the concentration was less than 500 µg/ml. Assays were always conducted with less than 250 µg of protein per ml for a period of 5 s. The rate was not linear for more than 5 s, even if the cell suspensions were diluted, and it appeared that this lack of linearity was related to a rapid dissipation of the artificial Δψ.

**Proton-motive force.** Samples (2 ml) of an exponentially growing culture were incubated anaerobically with [7-14]benzoate (1.00 µCi, 10 µCi/µmol), [1,2-14]polyethylene glycol (1.00 µCi, 0.1 µCi/mg), 3H2O (1.00 mCi, 0.25 µCi/mg), or tetraphenylphosphonium bromide (1.00 µCi, 30 to 40 µCi/µmol) for 5 min. Cultures were then transferred to microcentrifuge tubes (1.5 ml) which contained 0.35 g of silicon oil (equal-part mixture of Dexter Hysol 550 and Dexter Hysol 560; Hysol Co., Olean, N.Y.) and had been placed in an anaerobic glove box for 24 h. The tubes were centrifuged (13,000 x g, 5 min, 22°C), and 20-µl samples of supernatant were removed for scintillation counting.

The filters were then frozen (–15°C), and the bottoms containing cell pellets were removed with a pair of dog nail clippers. Pellets were dissolved in scintillation fluid and counted. Intracellular volume (1.5 µl/mg of protein) was computed from the difference in [14]polyethylene glycol and 3H2O. ΔpH was determined from the distribution of [14]benzoate between the medium and pellet by using the Henderson-Hasselbalch equation. Δψ was calculated from the uptake of [14C]tetraphenylphosphonium bromide according to the Nernst relationship. Nonspecific tetraphenylphosphonium bromide binding was estimated from cells which had been treated with valinomycin and nigericin (5 µM).

**Sodium and potassium.** Cells which had been centrifuged through silicon oil (as described above) were dissolved in 3 N HNO3 (24 h) and analyzed for K and Na with a Perkin-Elmer atomic absorption spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.). K and Na concentrations were calculated from the ratio of intracellular volume to protein (1.5 µl/mg), and these values were corrected for extracellular K and Na contamination. Na uptake was measured with a combination Na electrode (Microelectrodes Inc., Londonderry, N.H.).

**ATP determinations.** Cells (0.16 mg of protein) were extracted for 20 min in 0.5 ml of ice-cold 14% perchloric acid supplemented with 9 mM EDTA and then centrifuged (13,000 x g, 5 min, 22°C). A portion of the extract (1 ml) was neutralized with 0.5 ml of KOH-KHCO3 (1 M each) and quickly frozen. The neutralized extracts were thawed, centrifuged (13,000 x g, 5 min, 22°C), and assayed for ATP by the firefly luciferase method (15). Neutralized extracts were diluted 50-fold with 40 mM Tris containing 2 mM EDTA, 10 mM MgCl2, and 0.1% bovine serum albumin (pH 7.75). The luciferase reaction was initiated by adding 100 µl of a purified luciferase-luciferin mix to 100 µl of diluted extract according to the recommendations of the supplier (Sigma Chemical Co., St. Louis, Mo.). Light output was immediately measured with a luminometer (model 1250; LKB Instruments, Inc., Gaithersburg, Md.), and ATP served as the standard.

**Products.** Cell protein was measured by the method of Lowry et al. (14) after the cells had been heated to 100°C in 0.2 N NaOH for 15 min. Ammonia was assayed by the colorimetric method of Chaney and Marbach (4). Volatile fatty acids from metaphosphoric acid-treated (6% [wt/vol], final concentration) supernatant samples were measured with a Gow Mac model 580 flame ionization gas chromatograph equipped with a Supelco 1220 column (1% H3PO4, 100/120 mesh).

**Materials.** 14C-labeled amino acids were obtained from Amersham Corp., Arlington Heights, Ill. All chemicals and reagents were reagent grade.

**RESULTS**

Effect of sodium on growth and transport. When the ruminal peptostreptococcus was incubated in an Na-deficient medium with purified amino acids (1 g/liter) as a carbon source and 15 g of leucine per liter as an energy source, the cells grew very slowly (less than 0.02/h), and there was little increase in optical density (18-mm tubes, 600 nm) even after 24 h (Fig. 1). However, if NaCl (100 mM) was added, the bacteria grew more rapidly (0.17/h), and the final optical density at 24 h was more than 0.9 (Fig. 1).

When K-loaded cells were diluted into choline phosphate to create an artificial Δψ in absence of Na, no uptake of leucine was detected (Fig. 2). However, rapid transport of leucine was observed if NaCl or LiCl was added to choline phosphate, and transport could be driven by a chemical gradient of Na⁺ (ΔµNa⁺) in the absence of an artificial Δψ. An artificial ΔpH created by acetate diffusion could not serve as a driving force for leucine transport with or without Na. No transport was observed in the absence of either an artificial Δψ or a ΔµNa⁺. Similar results were obtained with isoleucine and valine (data not shown).

When the rate of leucine transport was measured at Na⁺ concentrations ranging from 0 to 100 mM, there was little increase at concentrations greater than 20 mM (Fig. 3b). An Eadie-Hofstee plot (v/S versus v) yielded a Kₘ for Na of 5.2 mM (Fig. 3a, inset), while a Hill plot indicated that the carrier had only one binding site for Na⁺ (Fig. 3b). Na⁺
FIG. 2. Transport of leucine by cells which were treated with valinomycin (5 μM), loaded with 100 mM potassium phosphate, and diluted 50-fold into phosphate buffers containing 100 mM choline (Δ) or 100 mM choline plus 100 mM NaCl (△). Cells were loaded with 100 mM potassium phosphate and diluted into 100 mM potassium phosphate plus 100 mM NaCl (■). Cells were loaded with 100 mM potassium phosphate plus 100 mM NaCl and were diluted into 100 mM sodium phosphate (△) or diluted into 100 mM potassium phosphate plus 100 mM NaCl (○). Cells were also loaded with 100 mM potassium phosphate and 80 mM acetate or with 100 mM potassium phosphate, 100 mM NaCl, and 80 mM acetate and were diluted into 100 mM potassium phosphate or 100 mM potassium phosphate plus 100 mM NaCl to create an artificial ΔpH in the absence or presence of Na, respectively (□). The pH was always 6.5.

uptake in the presence of leucine supported the assumption that leucine was transported in symport with Na⁺ (Fig. 4).

**Effects of pH on leucine transport.** When cells were treated with valinomycin, loaded with K (pH 6.5), and diluted into sodium phosphate at pH levels ranging from 5.0 to 7.0, there was little effect on leucine transport (Fig. 5a). Even at pH 4.5 the uptake rate decreased only 25%. However, if cells were loaded (30 min) at the same pH as they were diluted, there was a significant decline in the rate of uptake at pH levels less than 6.0 (Fig. 5b). Since the assay period over which a linear rate could be detected (5 s) was very short, the initial studies may not have reflected true changes in extracellular pH, intracellular pH, or the stability of the carrier.

**Isoleucine and valine.** When the peptostreptococcus was provided with 40 mM leucine, more than 70% was fermented during the 24-h incubation and the ratio of isovalerate to isocaproate was approximately 0.5 (Table 1). Little valine was deaminated, but if 10 mM leucine was also provided, isobutyrate production increased more than seven times and virtually all of the leucine was converted to isocaproate. When leucine was increased further, isobutyrate reached a maximal concentration of approximately 9 mM, some of the leucine was converted to isovalerate, and the cultures were no longer substrate limited. Total fermentation acid production was consistent with ammonia production. Based on theoretical pathways of branched-chain amino acid fermentation, the ratio of isobutyrate plus isovalerate to isocaproate should have been 0.5 (Fig. 6), a value which was in close agreement with the experimental results (Table 1). Similar experiments could not be conducted with isoleucine because standard methods of gas-liquid or high-pressure liquid chromatography are unable to separate isovalerate from 2-methylbutyrate.

**FIG. 3.** (a) Effect of added NaCl on leucine transport by cells loaded with potassium and diluted 50-fold into choline phosphate (pH 6.5). An Eadie-Hofstee (v/S versus v) plot is shown in the inset. (b) A Hill plot of the data shown.

When the peptostreptococcus was incubated with [14C]leucine and a 100-fold excess of nonradiolabeled glutamine, phenylalanine, serine, threonine, tryptophan, or alanine, little reduction of leucine transport (less than 10%) was observed (data not shown). Isoleucine and valine competitively inhibited leucine uptake (Fig. 7), and Kᵣ values for isoleucine and valine were 49 and 90 μM, respectively. There was some difference between the Vₘₐₓ and Kᵣ values

**FIG. 4.** Effect of leucine (1 mM) on the uptake of sodium by the ruminal peptostreptococcus cells (6.9 mg of cell protein) at pH 7.
for leucine, valine, and isoleucine (Table 2), but the $K_m$ to $V_{max}$ ratios were virtually the same (approximately 0.2 $\mu$M/nmol per mg of protein per min).

Effect of monensin. When the peptostreptococcus was incubated with 15 g of Casamino Acids per liter, monensin addition (5 $\mu$M) caused an almost immediate cessation of growth but ammonia production continued (data not shown). Since cells which were pretreated with monensin (5 $\mu$M) and incubated with 100 mM leucine produced nearly half as much ammonia as untreated controls, the inhibition of growth could not be explained solely on the basis of transport.

When untreated cultures (15 g of Casamino Acids per liter) were incubated with $[^{14}C]$tetraphenylphosphonium bromide or benzoate to estimate the proton-motive force ($\Delta\phi$), $\Delta\phi$ was 91 mV and there was little $Z\Delta pH$ (Table 3). Monensin caused a decrease in $K_+$, an increase in Na, a reversal of intracellular pH, and a small increase in $\Delta\phi$. The inhibition of growth was concomitant with a large decline in ATP.

**DISCUSSION**

When leucine was provided as the sole energy source, the peptostreptococcus was unable to grow in the absence of Na and the lack of growth could be explained by the effect of Na on transport. Na dependency was supported by the observations that (i) leucine transport could be driven by an artificial $\Delta\psi$ only when Na was present, (ii) a chemical gradient of Na$^+$ (Na$^+$) alone was sufficient to drive transport, and (iii) Na$^+$ was taken up in the presence of leucine. Since $Z\Delta pH$ could not serve as a driving force even when Na was present, it appeared that leucine was transported by symport with Na$^+$ in response to either a $\Delta\psi$ or $\Delta\psi^+$.

**TABLE 1. Effect of leucine on the deamination and fermentation of valine by the ruminal peptostreptococcus**

<table>
<thead>
<tr>
<th>Valine (mM)</th>
<th>Leucine (mM)</th>
<th>Values (mM) for:</th>
<th>Ratio of IB + IV to IC $^a$</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Icb</td>
<td>IL</td>
</tr>
<tr>
<td>0</td>
<td>40</td>
<td>0</td>
<td>9.3</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>9.3</td>
<td>0.9</td>
</tr>
<tr>
<td>40</td>
<td>10</td>
<td>6.6</td>
<td>0.2</td>
</tr>
<tr>
<td>40</td>
<td>20</td>
<td>9.2</td>
<td>0.9</td>
</tr>
<tr>
<td>40</td>
<td>30</td>
<td>9.2</td>
<td>2.8</td>
</tr>
<tr>
<td>40</td>
<td>40</td>
<td>8.5</td>
<td>4.3</td>
</tr>
</tbody>
</table>

$^a$ Washed cells (150 mg of protein per liter) were incubated for 24 h with each of the substrates or combinations of substrates.

$^b$ Ratio of isobutyrate (IB) plus isovalerate (IV) to isocaproate (IC).

**FIG. 6.** Theoretical pathways and stoichiometries of branched-chain amino acid fermentation by the ruminal peptostreptococcus.

**FIG. 7.** An Eadie-Hofstee plot of leucine transport with either isoleucine or valine as competitive inhibitors. Isoleucine and valine were provided at 50 $\mu$M, and the $K_i$ values were 49 and 90 $\mu$M, respectively.
TABLE 2. The Michaelis constants of the branched-chain amino acid carrier which were estimated from an Eadie-Hofstee plot and the square of the regression coefficient ($r^2$)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (nanomoles per milligram of protein per minute)</th>
<th>Ratio of $K_m$ to $V_{max}$</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>77</td>
<td>328</td>
<td>0.23</td>
<td>0.93</td>
</tr>
<tr>
<td>Valine</td>
<td>55</td>
<td>243</td>
<td>0.22</td>
<td>0.93</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>33</td>
<td>194</td>
<td>0.17</td>
<td>0.94</td>
</tr>
</tbody>
</table>

* Ratios are given as micromolars per (nanomoles per milligram of protein per minute).

kinetics are not confounded by intracellular metabolism. We were able to prepare membrane vesicles of the peptostreptococcus, but the length of time needed for protoplast formation was very long (>1.5 h), even if the cultures were pretreated with penicillin. Since previous work showed that the peptostreptococcus contained 80% protein (5), it is not all that surprising that the cell wall was resistant to lysozyme and mutanolysin. Some osmotically sensitive vesicles were obtained, but they were unable to transport branched-chain amino acids via a K diffusion potential (data not shown). This inactivity could have been related to the time needed for protoplast formation and exposure to hydrolytic enzymes.

Previous studies showed that the ruminal peptostreptococcus deaminated leucine at a very rapid rate and that isoleucine and valine were fermented slowly (5). However, leucine, isoleucine, and valine were all taken up rapidly by a common carrier, and differences in ammonia production could not be explained by a transport limitation. Leucine is deaminated by a dual pathway in which reducing equivalents resulting from isovalerate formation are used in isocaproate production (Fig. 6). Valine and isoleucine are converted to isobutyrate and 2-methylbutyrate, respectively, by pathways which contain two sites that generate reducing equivalents and furnish no means of reducing-equivalent disposal. When leucine was provided with valine, reducing equivalents arising from isobutyrate production were used in isocaproate formation, and the rate of valine deamination increased markedly (Table 1). Based on the observation that this species produces small amounts of hydrogen and that interspecies hydrogen transfer to ruminal methanogens provides another means of reducing-equivalent disposal (3), the ruminal peptostreptococcus could play an important role in the degradation of ruminal valine and isoleucine as well as leucine.

Continuous culture studies indicated that the peptostreptococcus was sensitive to growth at low pH (5), and this sensitivity was consistent with the effect of pH on transport (Fig. 2b). It is well documented that low pH in the rumen is detrimental to the growth of ruminal cellulolytic bacteria (23). While the direct effect of pH on cellulolytic bacteria cannot be discounted (19), branched-chain volatile fatty acid requirements should be considered. The negative impact of low pH on the peptostreptococcus could lead to a deficiency of branched-chain volatile fatty acids and an inhibition of cellulolysis.

Monensin is an ionophore which is effective primarily against gram-positive bacteria (18). For many years it was assumed that *Megasthphaera elsdenii* was the primary producer of ruminal branched-chain volatile fatty acids (16), but its resistance to monensin (8) could not explain the negative relationship between monensin and decreased branched-chain volatile fatty acids (17). The peptostreptococcus was sensitive to monensin, and this sensitivity may explain the observation that exogenous branched-chain volatile fatty acids overcame the negative effect of monensin on milk production (L. L. Coutinho, P. F. Machado, R. M. Cook, Abstr. 82nd Annu. Meet. Am. Dairy Sci. Assoc. 1987, abstr. no. P339, p. 217).

The effect of monensin on the peptostreptococcus was consistent with a general mechanism of ionophore action in ruminal bacteria (20). K was depleted, and there was an influx of H+ (reversal of ΔpH) and subsequent increase in Na (Table 3). The Na content of untreated cells was very high (>600 mM), but recent work showed that *S. bovis*, another ruminal bacterium, also had a large pool of bound Na (24). Since an ionophore should have little effect on Na binding to the cell wall, the Na+ gradient was probably reversed by monensin. A reversed Na+ gradient across the cell membrane could have impeded leucine transport and ammonia production, but Δψ, another driving force for uptake, was not significantly affected by the electroneutral action of this antipporter. Growth inhibition could most easily be explained by a large decrease in ATP and excessive ATPase activity which would be needed to expel incoming H+ or Na+.

TABLE 3. The effect of monensin on the membrane potential, ATP, and intracellular cations of the ruminal peptostreptococcus

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values for:</th>
</tr>
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<td></td>
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<tr>
<td>$\Delta$ψ (mV)</td>
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</tr>
<tr>
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</tr>
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<td>Na (mM)*</td>
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<tr>
<td>Na (mM)*</td>
<td>622</td>
</tr>
<tr>
<td>ATP (mM)</td>
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</tr>
</tbody>
</table>

* Cells were grown with 15 g of Casamino Acids per liter. Control cells were harvested at an optical density of 0.68. Monensin-treated cells were harvested 1 h later at a similar optical density.

ACKNOWLEDGMENT

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LITERATURE CITED