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Amino Acid Transport in the Thermophilic Anaerobe *Clostridium fervidus* Is Driven by an Electrochemical Sodium Gradient

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Amino acid transport was studied in membranes of the peptidolytic, thermophilic, anaerobic bacterium *Clostridium fervidus*. Uptake of the negatively charged amino acid L-glutamate, the neutral amino acid L-serine, and the positively charged amino acid L-arginine was examined in membrane vesicles fused with cytochrome *c*-containing liposomes. Artificial ion diffusion gradients were also applied to establish the specific driving forces for the individual amino acid transport systems. Each amino acid was driven by the $\Delta\Psi$ and $\Delta\bar{\mu}_{Na^+}/F$ and not by the $Z\Delta pH$. The Na^+ stoichiometry was estimated from the amino acid-dependent $^{22}Na^+$ efflux and Na^+-dependent ^3H-amino acid efflux. Serine and arginine were symported with 1 Na^+ and glutamate with 2 Na^+. *C. fervidus* membranes contain Na^+/Na^+ exchange activity, but Na^+/H^+ exchange activity could not be demonstrated.

Bacteria growing in nonextreme environments pump out H^+ to keep the intracellular pH around 7 to 8 and maintain an inwardly directed Na^+ concentration gradient and an outwardly directed K^+ gradient (1, 15, 32, 41). These H^+ and Na^+ concentration gradients in turn can be used for energetically unfavorable reactions, such as motility and uphill transport of substrates (10, 32, 33). In bacteria, secondary transport often occurs in symport with H^+, but in recent years several Na^+ symport systems have also been described (26, 37). Na^+ taken up into the cell by symport systems can be pumped out again by a Na^+/H^+ antiporter or, less commonly, by a primary sodium pump, such as decarboxylases and ATPases in anaerobic bacteria and components of the electron transport chain in aerobic, marine bacteria (10–12, 33, 38).

Bacteria living in extreme environments have adapted to their surroundings in various ways. Alkaliphilic bacteria, growing at high pH, have an outwardly directed H^+ gradient maintained by an electrogenic Na^+/H^+ antiporter. Secondary transport in these organisms is driven by the $\Delta\tilde{\mu}_{Na^+}/F$ (20–22), where F is the Faraday constant. Acidophilic bacteria, growing at low pH values, use H^+ as a chemiosmotic coupling ion. The very large, inwardly directed H^+ gradient is compensated by a reversed membrane potential (22, 27). Thermophilic bacteria are adapted to elevated temperatures by compensating the increased permeability for ions. In general, thermophiles have altered their membrane composition so that at the same temperature the membranes are more rigid and less fluid than the membranes of mesophiles (23). The membrane of the aerobic thermophile *Bacillus stearothermophilus* is, at its growth temperature (65°C), equally viscous as its mesophilic counterpart *Bacillus subtilis* at its growth temperature (37°C); however, the H^+ permeability is much higher (5, 8). *B. stearothermophilus* compensates the increased H^+ permeability by investing more metabolic energy in very active H^+ pumps present in its membrane (5, 8). Secondary transport systems in this organism are Na^+ symport systems, except for glutamate, which is symported together with a H^+ and Na^+ ion, and lysine, which is transported via a uniport mechanism (6, 18). For anaerobic thermophilic bacteria, which derive relatively little metabolic energy from their substrates and produce ΔpH dissipating fatty acids, the mechanism of energy-transducing processes is not well established but may very well differ from that in the aerobes.

In this study, amino acid transport was studied in *Clostridium fervidus*, a fermentative, peptidolytic, and thermophilic organism. The role of Na^+ as a coupling ion in transport of the neutral amino acid serine, the negatively charged amino acid glutamate, and the positively charged amino acid arginine was examined in detail. Under all conditions tested, amino acid transport was found to be dependent on the $\Delta\tilde{\mu}_{Na^+}/F$ and without involvement of the $Z\Delta pH$ as a driving force ($Z = 2.3\ RT/F = 58.8$ at room temperature, where R = gas constant and T = absolute temperature). The bioenergetic implications of this phenomenon are discussed.

MATERIALS AND METHODS

Cell growth and preparation of membrane vesicles. *C. fervidus* ATCC 43204 was grown at 68°C in the tryptone-yeast extract-glucose (TYEG) medium, and membrane vesicles were prepared as described by Speelmans et al. (35).

Purification and reconstitution of *B. stearothermophilus* cytochrome *c* oxidase. The purification of *B. stearothermophilus* cytochrome *c* oxidase was performed as described by de Vrij et al. (7), with omission of the final gel filtration step. The oxidase preparation contained 12.7 nmol of heme *a* per mg of protein and was stored in liquid nitrogen. Proteoliposomes containing *B. stearothermophilus* cytochrome *c* oxidase were prepared essentially as described by Hinkle et al. (19). *B. stearothermophilus* cytochrome *c* oxidase was reconstituted into liposomes consisting of acetone-ether-washed *Escherichia coli* phospholipid and L-phosphatidylcholine (3:1 [wt/wt]) at a ratio of 0.4 nmol of heme *a* per mg of phospholipid.

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Preparation of liposomes and fusion of (proteo)liposomes with *C. fervidus* membranes. Liposomes were prepared from acetone-ether-washed *E. coli* phospholipid and L-phosphatidylcholine dissolved in $CHCl_3$ in a 3:1 (wt/wt) ratio as described by Speelmans et al. (35). *C. fervidus* membrane vesicles (1 mg of membrane protein) were fused with liposomes or *B. stearothermophilus* cytochrome *c* oxidase-containing liposomes (10 mg of phospholipid) as described by Speelmans et al. (35), yielding hybrid membranes.

Determination of transmembrane gradients. The membrane potential (interior negative) generated by *B. stearothermophilus* cytochrome *c* oxidase was determined from the distribution of the tetraphenylphosphonium ion across the membrane with a tetraphenylphosphonium ion-selective electrode. The membrane potential was estimated by applying a correction for concentration-dependent probe binding as described by Lolkema et al. (24). Tetraphenylphosphonium ion uptake in hybrid membranes was registered at 30°C in a 1-ml vessel containing hybrid membranes (40 μg of protein per ml) and an air-saturated 50 mM 2(*N*-morpholino)ethanesulfonic acid (MES)–piperazine-*N*,*N*′-bis(2-ethanesulfonate) (PIPES)–*N*-2-hydroxyethylpiperazine-*N*′-2-morpholino-ethanesulfonic acid (HEPES)–NaOH buffer, containing 10 mM $MgSO_4$, adjusted to pH 6.0, 7.0, or 8.0 with *N*-methylglucamine. Tetraphenylphosphonium ion (4 μM) and the electron donor system ascorbate (20 mM) and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylene-diamine (TMPD) (300 μM) were added. The ionophore monensin (final concentration, 20 nM) and the uncoupler 3,5-di-*tert*-butyl-4-hydroxybenzelidene malonitrile (SF-6487) (final concentration, 300 nM) were used to convert the ZΔpH into $\Delta\bar{\mu}_{Na^+}/F$ and to dissipate the Δp, respectively.

Qualitative estimations of the artificially imposed membrane potential in the hybrid membranes were determined with the membrane potential indicator 3,3′-dipropylthiocarbocyanine iodide by measuring membrane potential-dependent absorbance changes ($\Delta A_{683-660}$) at 40°C as described by de Vrij et al. (5). The imposed ZΔpH was estimated by the method described by Clement and Gould (2).

Uptake of amino acids in hybrid membranes. The uptake of amino acids in hybrid membranes driven by a Δp, generated by *B. stearothermophilus* cytochrome *c* oxidase, was measured at 40°C by the described procedures (35). The buffer used was the same as described above. After 1.5 min of preincubation with the electron donor system ascorbate (20 mM) and TMPD (300 μM), [^{14}C]serine (final concentration, 5.8 μM), [^{14}C]glutamate (final concentration, 3.5 μM), or [^{14}C]arginine (final concentration, 4.4 μM) was added, and at given intervals samples of 100 μl were taken, diluted into 2 ml of ice-cold 0.1 M KCl, and filtered immediately over 0.45-μm-pore-size cellulose-nitrate filters (Millipore). Filters were washed once with 2 ml of 0.1 M KCl and transferred to scintillation vials. Scintillation fluid was added, and the radioactivity was measured with a liquid scintillation counter (Packard Tri-Carb 460 CD; Packard Instruments Corp.).

Uptake of amino acids in membranes driven by artificial gradients. Hybrid membranes obtained from vesicles fused with liposomes were loaded and concentrated in a buffer containing 20 mM (each) MES, PIPES, and HEPES, 100 mM potassium acetate, 10 mM $MgSO_4$, and various amounts (0 to 50 mM) of NaCl. The ionic strength was kept constant, and the pH was adjusted to 6.0, 7.0, or 8.0 with *N*-methylglucamine or H_2SO_4. Valinomycin was added to the hybrid membranes at a final concentration of 2 nmol/mg of protein. A combination of a membrane potential (ΔΨ) and a chemical gradient of sodium ($\Delta\bar{\mu}_{Na^+}/F$) was generated by diluting the hybrid membranes (loaded with a Na^+-free and K^+-containing buffer) 100-fold into a buffer containing 20 mM (each) MES, PIPES, and HEPES, 100 mM sodium acetate, and 10 mM $MgSO_4$. A chemical gradient of sodium ions only was generated by omitting the valinomycin. A membrane potential only was generated by dilution of valinomycin-treated hybrid membranes (loaded with a buffer containing various concentrations of Na^+) into a buffer containing 20 mM (each) MES, PIPES, and HEPES, 100 mM acetic acid, the same concentration of Na^+, and 10 mM $MgSO_4$. Dilution of the hybrid membranes into a buffer containing 20 mM (each) PIPES and HEPES, 100 mM potassium, 120 mM MES, the same amount of Na^+, and 10 mM $MgSO_4$ resulted in the generation of a transmembrane pH gradient (ZΔpH) only. As a control hybrid, membranes were diluted into the loading buffer (no gradient). In all cases the dilution buffer (200 μl) was supplemented with L-U-^{14}C-amino acids at final concentrations of 2.9 μM serine, 2.2 μM arginine, and 1.8 μM glutamate. The uptake experiments were performed at 40°C. At given intervals samples were taken, washed, rapidly filtered, and counted as described above.

Efflux, exchange, and counterflow experiments. Membrane vesicles were washed and resuspended in a 50 mM *N*-methylglucamine–MES buffer, pH 6.0, containing 5 mM $MgSO_4$ to final protein concentrations of 20 to 30 mg/ml. SF-6847 was added to a final concentration of 1 μM. The membrane vesicles were loaded with ^{3}H-amino acids (1 mM, 0.1 mCi/ml) and/or $^{22}Na^+$ (1 mM, 20 μCi/ml) for 3 h at 40°C. Membrane vesicles (2 μl) were diluted 300-fold into the equilibration buffer without Na^+ and amino acids (efflux) or with 1 mM NaCl and 1 mM amino acid (exchange). When Na^+/H^+ activity measurements were performed, SF-6847 was replaced by valinomycin (2 nmol/mg of protein), and the buffers used to generate an artificially ΔΨ and/or ZΔpH were composed as described above, with the pH adjusted to 6.5, 7.0, or 7.5. For the arginine-ornithine counterflow experiments, hybrid membranes were loaded with 1 mM unlabelled arginine or ornithine in a 50 mM sodium phosphate buffer and diluted 100-fold into the equilibration buffer, containing 1.7 μM [^{14}C]ornithine or 2.2 μM [^{14}C]arginine. At given times, 2 ml of ice-cold equilibration buffer was added and the samples were filtrated immediately over cellulose acetate filters. Filters were washed and counted as described above.

Protein determination. Protein was determined by the method of Lowry et al. (25) or Smith et al. (34) with bovine serum albumin as a standard.

Internal volume. The internal volume of the fused membranes was determined from the relative fluorescence quenching by cobalt of membrane-entrapped calcein as described by Oko et al. (29). An internal volume of 8 μl/mg of membrane protein was determined for the fused membranes. An internal volume of 3 μl/mg of membrane protein was used for *C. fervidus* membrane vesicles.

Materials. The following uniformly labelled ^{14}C-amino acids were used: L-serine (6.4 TBq/mol), L-glutamate (10.5 TBq/mol), L-arginine (12.6 TBq/mol), and L-ornithine (10.4 TBq/mol). The following uniformly labelled ^{3}H-amino acids were used: L-serine (1.07 TBq/mmol), L-glutamic acid (1.85 TBq/mmol), and L-arginine (2.29 TBq/mmol). $^{22}Na^+$ was at 27.2 TBq/mol. All radioactive chemicals were obtained from the Radiochemical Centre, Amersham, United Kingdom. All other chemicals were reagent grade.

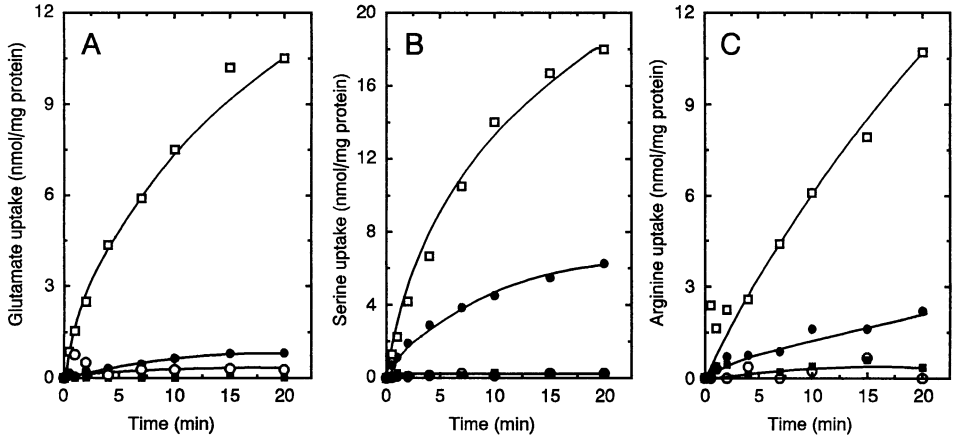

FIG. 1. Uptake of amino acids in cytochrome *c* oxidase-containing hybrid membranes of *C. fervidus*. Amino acid uptake was measured at 40°C in a 50 mM (each) MES-PIPES-HEPES buffer, containing 50 mM NaOH and 10 mM $MgSO_4$, set to pH with *N*-methylglucamine (as indicated below). Amino acid uptake was measured in the absence of an electron donor (○) or presence (●) of 20 mM K-ascorbate and 300 μM TMPD with the addition of 20 nM monensin (□) or 300 nM SF-6847 (■). (A) Glutamate (3.5 μM) uptake at pH 8.0; (B) serine (5.8 μM) uptake at pH 7.0; (C) arginine (4.4 μM) uptake at pH 8.0.

RESULTS

Amino acid transport in hybrid membranes obtained by fusion of membrane vesicles with cytochrome *c* oxidase-containing liposomes. Uptake of the amino acids L-glutamate, L-serine, and L-arginine (negatively charged, neutral, and positively charged at the pH values used) was studied in membrane vesicles of *C. fervidus* fused with liposomes containing cytochrome *c* oxidase from *B. stearothermophilus*. The membrane potential ($\Delta\Psi$) generated on the addition of ascorbate and TMPD to a Na^+-MES-PIPES-HEPES buffer was −124, −143, and −150 mV at pH 6.0, 7.0, and 8.0, respectively. On addition of monensin, the $\Delta\Psi$ amounted to −150 mV at all pH values. Δp-driven uptake of the different amino acids was observed, though the uptake of glutamate was relatively low (Fig. 1). Without ascorbate-TMPD or when the protonophore SF-6847 was present, accumulation of the amino acids was not observed (Fig. 1). The highest uptake was found in the presence of the Na^+/H^+ exchanger monensin. The same trends were found for all three amino acids at pH 6.0, 7.0, and 8.0 (data not shown). These results indicate that glutamate, serine, and arginine uptake is driven by (a component of) the Δp but that glutamate uptake is mainly dependent on a concentration gradient of sodium ions.

Amino acid uptake driven by artificial ion diffusion gradients. To establish the precise nature of the driving forces for glutamate, serine, and arginine transport, amino acid uptake in response to artificially imposed ion gradients was studied. At 40°C, the artificially imposed $\Delta\Psi$ in hybrid membranes was transient. After 2 min, the $\Delta\Psi$ had dropped to 50% of the initial value (data not shown), which is sufficiently stable to study qualitatively the nature of the driving forces in transport processes. The pH or the composition of the buffer had only minor effects on the magnitude and stability of the $\Delta\Psi$ imposed (data not shown). A $\Delta\Psi$, a ZΔpH, a $\Delta\bar{\mu}_{Na^+}/F$, or combinations of $\Delta\Psi$ and $\Delta\bar{\mu}_{Na^+}/F$ were employed. Glutamate was taken up in the presence of a sodium gradient. A small, but significant, uptake was observed when a $\Delta\Psi$ was applied in the presence of equal concentrations of sodium on the inside and outside. When a combination of both gradients was applied, the highest uptake was observed. No uptake was observed when a ZΔpH was applied in the presence of sodium (Fig. 2A). Serine uptake was found with a $\Delta\bar{\mu}_{Na^+}/F$ and a $\Delta\Psi$ alone (provided that Na^+ was present in the medium) and was most prominent when both gradients were presented (Fig. 2B). Strikingly, the uptake of arginine was driven by a $\Delta\Psi$ and by a sodium gradient. Again, the highest uptake was found when both gradients were present (Fig. 2C). No uptake of serine and arginine was found with ZΔpH as the sole driving force (data not shown). The same trends were observed at pH 6.0, 7.0, and 8.0 (data not shown). Serine and arginine uptake rates were highest (100%) at pH 6.0, but at pH 8.0 the rates were still 70%. The highest rate of glutamate uptake was found at pH 8.0. At pH 6.0, the uptake rate was only 55%. These results indicate that the uptakes of glutamate, serine, and arginine are electrogenic processes with Na^+ as a symported ion. The contribution of $\Delta\Psi$ as a driving force in the uptake of glutamate is less pronounced than with the other amino acids. The cause of this difference was further investigated.

Na^+ dependency of the glutamate transport system. Artificially imposed ion gradients were used to study uptake of L-glutamate in the presence of increasing amounts of Na^+ (inside and outside). The optimum concentration of Na^+ for $\Delta\Psi$-driven glutamate uptake was 10 mM (Fig. 3). At higher concentrations, the initial uptake rate is lowered. A ZΔpH alone could not drive glutamate uptake, irrespective of the absolute Na^+ concentrations. Since glutamate is negatively charged and a $\Delta\Psi$ can drive glutamate uptake, these results indicate that glutamate is taken up in symport with at least 2 Na^+, and that high internal Na^+ concentrations inhibit glutamate uptake. When glutamate uptake was measured in hybrid membranes containing cytochrome *c* oxidase (as in Fig. 1) with 10 mM NaCl inside and outside, Δp-driven uptake could be also demonstrated (data not shown).

Cation and substrate specificity of the amino acid transport systems. The cation specificity of the amino acid uptake systems was determined by imposing different ion concentration gradients in the presence of an artificial $\Delta\Psi$. Serine and arginine uptake could be driven by a concentration gradient of Na^+ or Li^+, but not by a NH_4^+, Rb^+, Cs^+, or choline$^+$ gradient ([outside] > [inside]). Glutamate uptake, on the other hand, could only be driven by a Na^+ gradient (Fig. 4).

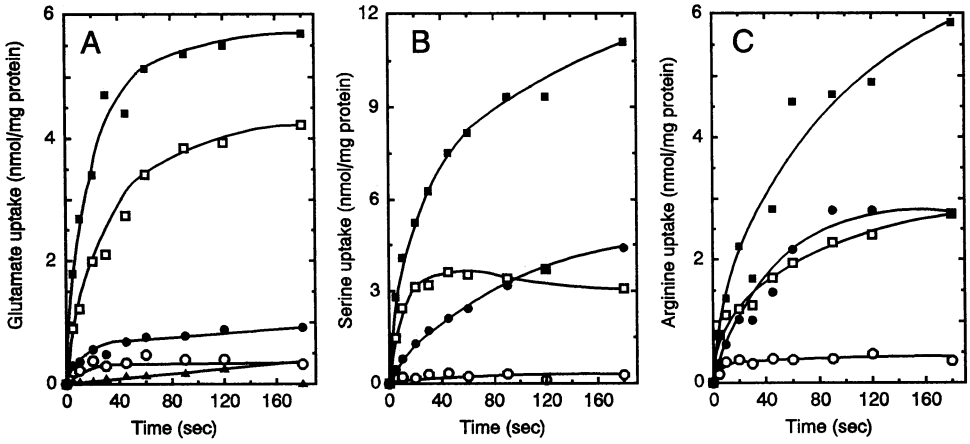

FIG. 2. Uptake of amino acids in hybrid membranes of *C. fervidus* driven by artificial ion gradients. Ion gradients were imposed as described in Materials and Methods, and experiments were performed at 40°C. Amino acid uptake was measured upon generation of a $\Delta\bar{\mu}_{Na^+}/F$ (□), a $\Delta\Psi$ in the presence of Na^+ (●), a $Z\Delta pH$ in the presence of Na^+ (▲), a $\Delta\Psi$ and a $\Delta\bar{\mu}_{Na^+}/F$ (■) or no gradient (○). (A) Glutamate (1.8 μM) uptake, pH 6.0; (B) serine (2.9 μM) uptake, pH 6.0; (C) arginine (2.2 μM) uptake, pH 8.0.

The substrate specificity of the L-glutamate uptake system was studied by measuring the initial rate of uptake in the presence of a 100-fold excess of unlabelled aspartate, glutamine, asparagine, cysteate, methylglutamate, hydroxyaspartate, succinate, or D-glutamate. None of these acids affected significantly the initial rate of uptake (data not shown). L-Serine uptake was completely inhibited by L-threonine and to a minor extent (60%) by L-alanine, L-cysteine, L-leucine, and glycine but not by D-serine (data not shown). Arginine uptake was inhibited by ornithine, and ornithine was inhibited by arginine (Fig. 5, insets). Also, arginine uptake was inhibited by lysine (data not shown).

Counterflow experiments showed that internal arginine or ornithine could be exchanged for external ornithine or arginine with similar rates (Fig. 5). The highest rates of transport were achieved in a buffer containing Na^+; in a Na^+-free buffer, uptake was about 20% (data not shown). Also, in exchange experiments with equimolar concentrations of arginine and ornithine inside and outside, exchange of the amino acids occurred at a higher rate in the presence of Na^+ (data not shown).

Stoichiometry of amino acid transport. Only serine was taken up to steady-state accumulation levels in cytochrome *c* oxidase-containing hybrid membranes. The cation/serine stoichiometry could, therefore, be determined. When only a $\Delta\Psi$ is the driving force ($[Na^+]_{in} = [Na^+]_{out}$), steady-state levels of accumulation are $\Delta\bar{\mu}_{serine}/F = n\Delta\Psi$, where n represents the cation-serine stoichiometry during transport. The stoichiometry was 0.9 cations per serine (35). For arginine uptake, a steady-state level of accumulation was not reached after 20 min, while for glutamate the inhibitory role of the internal Na^+ complicates the thermodynamic analysis of amino acid/Na^+ stoichiometries. Therefore, kinetic experiments in which the Na^+/amino acid stoichiometry was estimated directly from the Na^+-stimulated ^3H-amino acid efflux and the amino acid-stimulated $^{22}Na^+$ efflux were performed. Figure 6 shows the typical result obtained for glutamate. The ratio of the rates of amino acid-stimulated Na^+ efflux and Na^+-stimulated amino acid efflux gives the Na^+/amino acid stoichiometry. These ratios were 1.0 for Na^+ to serine, 1.1 for Na^+ to arginine, and 1.9 for Na^+ to glutamate. With this method, rather high variations in the

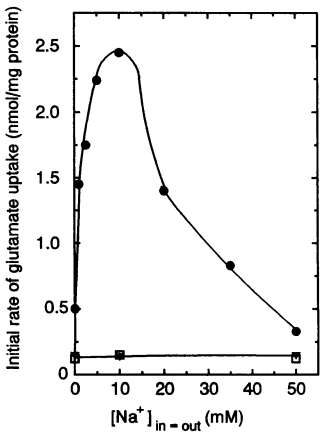

FIG. 3. Effect of Na^+ concentration on the uptake of glutamate in hybrid membranes of *C. fervidus*. Uptake was performed at 40°C as described in Materials and Methods. The pH was set at 6.0, and the glutamate concentration used was 1.8 μM. The internal $[Na^+]$ equaled the external $[Na^+]$. An artificial $\Delta\Psi$ (●), an artificial $Z\Delta pH$ (□), or no gradient (▼) was applied.

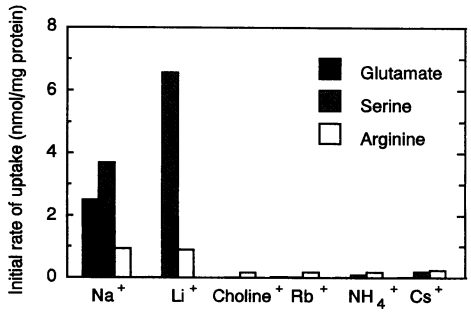

FIG. 4. Cation specificity of the amino acid uptake systems in hybrid membranes of *C. fervidus*. Uptake was performed at 40°C, pH 7.0, on imposition of valinomycin-mediated K^+ diffusion potential. The external medium contained 50 mM NaCl, LiCl, choline Cl, RbCl, NH_4Cl, or CsCl. Glutamate (1.8 μM) uptake, serine (2.8 μM) uptake, and arginine (2.2 μM) uptake were measured.

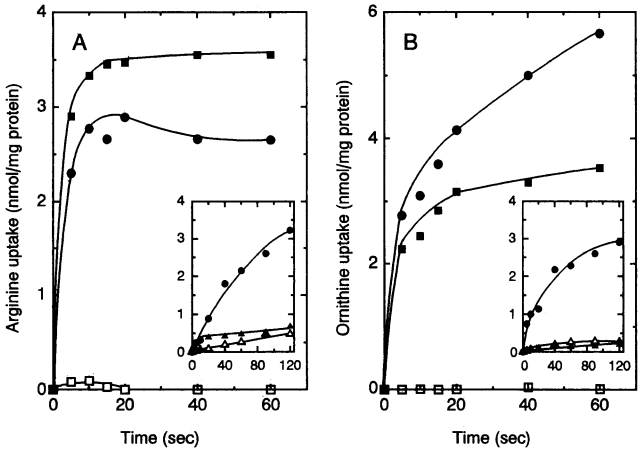

FIG. 5. Arginine/ornithine counterflow in hybrid membranes of *C. fervidus*. (A) Membranes were loaded with 1 mM ornithine (■), 1 mM arginine (●), or nothing (□) and diluted into a buffer containing 2.2 μM [^{14}C]arginine. (B) Membranes were loaded with 1 mM ornithine (●), 1 mM arginine (■), or nothing (□) and diluted into a buffer containing 1.7 μM [^{14}C]ornithine. The inset of panel A shows uptake of arginine, and the inset of panel B shows uptake of ornithine, driven by a $\Delta\Psi$ and $\Delta\bar{\mu}_{Na^+}/F$ without further additions (●) and in the presence of a 100-fold excess of unlabelled arginine (△) or ornithine (▲).

apparent Na^+/glutamate stoichiometries were observed (Table 1).

$^{22}Na^+$ exchange experiments. Since in bacteria the most common mechanism to generate a Na^+ gradient ([outside] > [inside]) is the interconversion of a Δp into a $\Delta\bar{\mu}_{Na^+}/F$, the presence of a Na^+/H^+ exchange system was examined. Membrane vesicles loaded with $^{22}Na^+$ showed no enhanced efflux at pH 7.0 when a $\Delta\Psi$, a $Z\Delta pH$, or a combination of both gradients was applied (data not shown). At pH 6.5, 7.0, or 7.5, an imposed Δp as high as −240 mV did not significantly enhance Na^+ efflux in membrane vesicles or in hybrid membranes. On the other hand, when externally an equimolar concentration of Na^+ was present, enhanced efflux was observed. Li^+ could replace Na^+ (Fig. 7). However, Na^+/H^+, Na^+/K^+, Na^+/Ca^{2+}, or Na^+/NH_4^+ exchange activities could not be demonstrated.

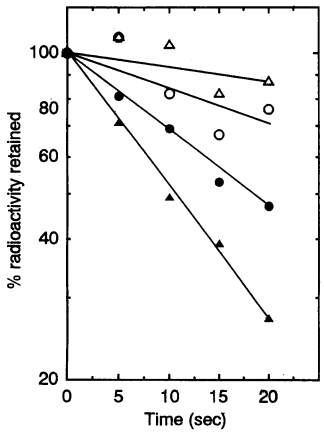

FIG. 6. Efflux of $^{22}Na^+$ and [^{3}H]glutamate from *C. fervidus* membrane vesicles. Membrane vesicles were loaded with 1 mM $^{22}Na^+$, 1 mM [^{3}H]glutamate, or both for 3 h at 40°C. The equilibration buffer contained 50 mM MES buffer, plus 5 mM $MgSO_4$, set at pH 6.0 with *N*-methylglucamine. Membrane vesicles (2 μl) were diluted 300-fold into the equilibration buffer. Efflux experiments were performed at 40°C. Efflux of $^{22}Na^+$ without (△) and together with (▲) 1 mM [^{3}H]glutamate and efflux of [^{3}H]glutamate without (○) and together with (●) 1 mM $^{22}Na^+$ were estimated. Samples were determined in fourfold.

TABLE 1. Stoichiometry of Na^+-coupled amino acid efflux from membranes of *C. fervidus*[a]

Amino acid	$\bar{x}$	SD	*n*
Glutamate	1.9	0.7	11
Serine	1.0	0.05	4
Arginine	1.1	0.03	4

[a] Efflux experiments were performed as described in the legend to Fig. 6. Experiments were performed in membrane vesicles, in membrane vesicles fused with liposomes, and in liposomes in which membrane proteins were reconstituted. $\bar{x}$, mean value of the Na^+/amino acid stoichiometry; *n*, number of experiments performed.

DISCUSSION

This paper shows that the peptidolytic, thermophilic anaerobic bacterium *C. fervidus* takes up amino acids in symport with Na^+. No secondary transport systems with protons as coupling ions could be demonstrated. Also, the presence of a Na^+/H^+ exchanger could not be demonstrated in membrane vesicles of this organism, despite the high sensitivity of the method used.

The neutral amino acid serine was found to be symported with 1 Na^+. A $\Delta\Psi$ and a $\Delta\bar{\mu}_{Na^+}/F$ are equally effective as the driving force for the uptake, and efflux of 1 mol of serine is coupled to the efflux of 1 mol of Na^+. Also, in *E. coli* a Na^+ symport system has been found for serine and threonine, albeit with a much higher affinity for Na^+ (16). Besides a Na^+-coupled system, a serine/H^+ symport system is present in *E. coli* (17). In *Streptococcus bovis* a serine (threonine)/Na^+ symport system with a low affinity for Na^+ has been found. However, in this case Li^+ cannot replace Na^+ (30).

Arginine is a positively charged amino acid. Uptake via a uniport system with the $\Delta\Psi$ as the driving force is in

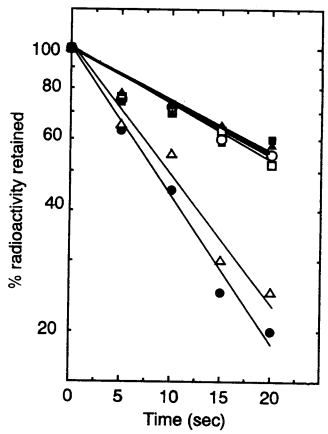

FIG. 7. Efflux of $^{22}Na^+$ from *C. fervidus* membranes. Membrane vesicles were loaded with 1 mM $^{22}Na^+$, and efflux was performed as described in the legend to Fig. 6 with, on the outside, 1 mM NaCl (●), 1 mM LiCl (△), 1 mM KCl (□), 1 mM NH_4Cl (▲), 1 mM $CaCl_2$ (■), or no salt (○).

principle possible, as has been shown for lysine transport in *B. stearothermophilus* (18). Arginine uptake in *C. fervidus* is symported with 1 Na^+ and therefore driven by $2\times \Delta\Psi$ and a $\Delta\bar{\mu}_{Na^+}/F$. Because of this high driving force for Na^+/arginine symport, a very high arginine concentration gradient would be expected in the cytochrome *c* oxidase-containing hybrid membranes. However, because of the relatively slow uptake of arginine, a steady-state accumulation level was not achieved. Efflux experiments, however, demonstrated unequivocally that arginine was transported with 1 Na^+. In whole cells of *C. fervidus*, the generation of citrulline from arginine could be measured, indicating that the arginine deiminase pathway is present (34a). This pathway is present in a number of bacteria, and per 1 mol of arginine, 1 mol of ATP, 1 mol of ornithine, 1 mol of CO_2, and 2 mol of NH_3 are formed (3). Arginine is taken up by the cells in exchange for ornithine, thereby minimizing the bioenergetic costs of transport. In *Lactococcus lactis* a true arginine/ornithine antiporter is present. No Δp-driven uptake of arginine via this carrier is possible (13). In *Pseudomonas aeruginosa* a Δp-driven uptake system of arginine, which functioned under physiological conditions as an arginine/ornithine exchanger, was present. It has not been established whether arginine was taken up in this organism via a uniport system or in symport with a proton (40). Our results demonstrate that in *C. fervidus*, arginine can be taken up in symport with 1 Na^+ and that under physiological conditions, arginine uptake takes place in exchange for intracellularly formed ornithine. This exchange is also Na^+ dependent. A Na^+/arginine symport system was also proposed for the rumen bacterium strain SR (39). It was suggested that ornithine efflux was also Na^+ coupled but mediated via a different carrier and that the $\Delta\bar{\mu}_{Na^+}/F$ generated by ornithine efflux could drive arginine uptake. However, it was not examined whether ornithine acts as a competitive inhibitor for arginine uptake and whether the carrier could perform arginine/ornithine exchange. The results obtained for the rumen bacterium could very well be explained by the model proposed for *C. fervidus*.

Glutamate is negatively charged at physiological pH values. The amino acid was taken up electrogenically in symport with 2 Na^+. A $\Delta\Psi$ and a $\Delta\bar{\mu}_{Na^+}/F$ drive the uptake of glutamate, and efflux of 1 mol of glutamate is coupled to efflux of, most likely, 2 mol of Na^+. In a previous article, glutamate was reported to be transported with 1 Na^+ (35). These experiments were performed under conditions of a high $[Na^+]_{in}$, which inhibited the uptake, and $\Delta\Psi$-driven uptake was not detected. Intracellular Na^+ ions may inhibit the uptake of glutamate by preventing the release of the Na^+ ions on the inside through a shift in the equilibrium of the carrier-ligand intermediate(s) to a form that has the Na^+-binding site(s) occupied. Serine uptake was also inhibited when high internal Na^+ concentrations were employed (data not shown). In the thermophilic aerobic bacterium *B. stearothermophilus*, glutamate is taken up in symport with 1 H^+ and 1 Na^+ (6). Such a Na^+/H^+/glutamate symport system was also reported for *E. coli* (9), but recently two distinct genes encoding two different secondary transport proteins were cloned (4). One of these genes (*gltP*) codes for a protein which takes up glutamate with, most likely, 2 H^+ (35a, 36); the other (*gltS*) codes for a Na^+/glutamate transport protein. The stoichiometry of the latter process has not been determined. The glutamate uptake system of *C. fervidus* is the first system for which it has been demonstrated that 2 Na^+ are cotransported with the amino acid. Like the GHS of *E. coli*, Li^+ cannot replace Na^+, but unlike this system, α-methylglutamate is not an inhibitor (31). The glutamate uptake system of *C. fervidus* also has a substrate specificity differing from that in *B. stearothermophilus*. The gene encoding the *C. fervidus* protein has been cloned and is currently being characterized genetically. It is of special interest to compare its primary structure with that of the other known glutamate transport proteins.

This study demonstrates that glutamate, arginine, and serine are $\Delta\bar{\mu}_{Na^+}/F$-driven symport systems in *C. fervidus*. This implies that even when there is a low Δp in the cells, the amino acids can be taken up as long as a $\Delta\bar{\mu}_{Na^+}/F$ is present. At the growth temperature of 68°C, the membranes are probably very leaky for protons. Furthermore, weak acids such as acetic acid are formed, which could lower the Δp by dissipating the $Z\Delta pH$ component. It is under those conditions certainly an advantage to have Na^+-coupled systems, provided the Na^+ gradient is generated by a primary Na^+ pump, since the permeability of the membrane for H^+ is orders of magnitude higher than that of Na^+ (28). The way in which this Na^+ gradient is generated in *C. fervidus* is not yet clear. Although Na^+/Na^+ exchange is observed, no Na^+/H^+ antiport activity could be demonstrated. In eukaryotes Na^+/Li^+ exchange activities, which do not result from a Na^+/H^+ antiporter and of which the physiological role is unclear, are also known (14). The presence of a Na^+ ATPase to generate a Na^+ gradient in *C. fervidus* seems most likely, so studies of the coupling ion of the membrane-bound ATPase are under way.

REFERENCES

1. **Booth, I.** 1985. Regulation of cytoplasmic pH in bacteria. Microbiol. Rev. **49:**359–378.
2. **Clement, N. R., and M. J. Gould.** 1981. Pyranine (8-hydroxy-1,3.6-pyrenotrisulfate) as a probe of internal aqueous hydrogen ion concentration in phospholipid vesicles. Biochemistry **20:** 1534–1538.
3. **Cunin, R., N. Glansdorff, A. Pierárd, and V. Stalon.** 1986. Biosynthesis and metabolism of arginine in bacteria. Microbiol. Rev. **50:**314–352.
4. **Deguchi, Y., I. Yamato, and Y. Anraku.** 1989. Molecular cloning of *gltS* and *gltP*, which encode glutamate carriers of *Escherichia coli* B. J. Bacteriol. **171:**1314–1319.
5. **de Vrij, W., R. A. Bulthuis, and W. N. Konings.** 1988. Comparative study of the energy-transducing properties of cytoplasmic membranes from a mesophilic and thermophilic *Bacillus* species. J. Bacteriol. **170:**2359–2366.
6. **de Vrij, W., R. A. Bulthuis, P. R. van Iwaarden, and W. N. Konings.** 1989. Mechanism of L-glutamate transport in membrane vesicles from *Bacillus stearothermophilus*. J. Bacteriol. **171:**1118–1125.
7. **de Vrij, W., R. I. R. Heyne, and W. N. Konings.** 1989. Characterization and application of a thermostable primary transport system: cytochrome-*c* oxidase from *Bacillus stearothermophilus*. Eur. J. Biochem. **178:**763–770.
8. **de Vrij, W., G. Speelmans, R. I. R. Heyne, and W. N. Konings.** 1990. Energy transduction and amino acid transport in thermophilic aerobic and fermentative bacteria. FEMS Microbiol. Rev. **75:**183–200.
9. **Dibrov, P. A.** 1991. The role of sodium ion transport in *Escherichia coli* energetics. Biochim. Biophys. Acta **1056:**209–224.
10. **Dimroth, P.** 1987. Sodium ion transport decarboxylases and other aspects of sodium ion cycling in bacteria. Microbiol. Rev. **51:**320–340.
11. **Dimroth, P.** 1990. Mechanisms of sodium transport in bacteria. Phil. Trans. R. Soc. Lond. B **326:**465–477.
12. **Dimroth, P.** 1991. Na^+-coupled alternative to H^+-coupled primary transport systems in bacteria. Bioessays **13:**463–468.
13. **Driessen, A. J. M., B. Poolman, R. Kiewiet, and W. N. Konings.** 1987. Arginine transport in *Streptococcus lactis* is catalyzed by a cationic exchanger. Proc. Natl. Acad. Sci. USA **84:**6093–6097.

14. **Escobales, N., and F. Figueroa.** 1991. Na^+/Na^+ exchange and Na^+/H^+ antiport in rabbit erythrocytes: two distinct transport systems. J. Membr. Biol. **120:**41–49.
15. **Futai, M., and T. Tsuchiya.** 1987. Proton transport and proton-motive force in prokaryotic cells, p. 3–83. *In* B. P. Rosen and S. Silver (ed.), Ion transport in prokaryotes. Academic Press, Inc., San Diego, Calif.
16. **Hama, H., T. Shimamoto, M. Tsuda, and T. Tsuchiya.** 1987. Properties of Na^+-coupled serine-threonine transport system in *Escherichia coli*. Biochim. Biophys. Acta **905:**231–239.
17. **Hama, H., T. Shimamato, M. Tsuda, and T. Tsuchiya.** 1988. Characterization of a novel L-serine transport system in *Escherichia coli*. J. Bacteriol. **170:**2236–2239.
18. **Heyne, R. I. R., W. de Vrij, W. Crielaard, and W. N. Konings.** 1991. Sodium ion-dependent amino acid transport in membrane vesicles of *Bacillus stearothermophilus*. J. Bacteriol. **173:**791–800.
19. **Hinkle, P. C., J. J. Kimm, and E. Racker.** 1972. Ion transport and respiratory control in vesicles formed from cytochrome oxidase and phospholipids. J. Biol. Chem. **247:**1338–1339.
20. **Krulwich, T. A., A. A. Guffanti, and D. Seto-Young.** 1990. pH homeostasis and bioenergetic work in alkalophiles. FEMS Microbiol. Rev. **75:**255–270.
21. **Krulwich, T. A., D. D. Hicks, D. Seto-Young, and A. A. Guffanti.** 1989. The bioenergetics of alkalophilic bacilli. Crit. Rev. Microbiol. **16:**15–36.
22. **Krulwich, T. A., and D. M. Ivey.** 1990. Bioenergetics in extreme environments, p. 417–448. *In* T. A. Krulwich (ed.), The bacteria, vol. XII. Bacterial energetics. Academic Press, Inc., San Diego, Calif.
23. **Langworthy, T. A.** 1982. Lipids of bacteria living in extreme environments. Curr. Top. Membr. Transp. **17:**45–77.
24. **Lolkema, J. S., K. J. Hellingwerf, and W. N. Konings.** 1982. The effect of 'probe binding' on the quantitative determination of the proton-motive force. Biochim. Biophys. Acta **681:**85–94.
25. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
26. **Maloy, S. R.** 1990. Sodium-coupled cotransport, p. 203–224. *In* T. A. Krulwich (ed.), The bacteria, vol. XII. Bacterial energetics. Academic Press, Inc., San Diego, Calif.
27. **Matin, A.** 1990. Keeping a neutral cytoplasm: the bioenergetics of obligate acidophiles. FEMS Microbiol. Rev. **75:**307–318.
28. **Nichols, J. W., and D. W. Deamer.** 1980. Net proton-hydroxyl permeability of large unilamellar liposomes measured by an acid-ase titration technique. Proc. Natl. Acad. Sci. USA **77:** 2038–2042.
29. **Oko, N., D. A. Kendall, and R. C. MacDonald.** 1982. A simple procedure for the determination of the trapped volume of iposomes. Biochim. Biophys. Acta **691:**332–340.
30. **Russell, J. B., H. J. Strobel, A. J. M. Driessen, and W. N. Konings.** 1988. Sodium-dependent transport of neutral amino acids by whole cells and membrane vesicles of *Streptococcus bovis*, a ruminal bacterium. J. Bacteriol. **170:**3531–3536.
31. **Schellenberg, G. D., and C. E. Furlong.** 1977. Resolution of the multiplicity of the glutamate and aspartate transport systems in *Escherichia coli*. J. Biol. Chem. **252:**9055–9064.
32. **Skulachev, V. P.** 1987. Bacterial sodium transport: bioenergetic function of sodium ions, p. 131–164. *In* B. P. Rosen and S. Silver (ed.), Ion transport in prokaryotes. Academic Press, Inc., San Diego, Calif.
33. **Skulachev, V. P.** 1989. The sodium cycle: a novel type of bacterial energetics. J. Bioenerg. Biomembr. **21:**635–648.
34. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. M. Gartner, M. D. Provenzano, E. K. Fujimoto, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150:**76–85.

34a. **Speelmans, G.** Unpublished data.

35. **Speelmans, G., W. de Vrij, and W. N. Konings.** 1989. Characterization of amino acid transport in membrane vesicles from the thermophilic fermentative bacterium *Clostridium fervidus*. J. Bacteriol. **171:**3788–3795.

35a. **Tolner, B.** Unpublished data.

36. **Tolner, B., B. Poolman, B. Wallace, and W. N. Konings.** 1992. Revised nucleotide sequence of the *gltP* gene, which encodes the proton-glutamate-aspartate transport protein of *Escherichia coli* K-12. J. Bacteriol. **174:**2391–2393.
37. **Tolner, B., M. E. van der Rest, G. Speelmans, and W. N. Konings.** 1992. Sodium coupled transport in bacteria, p. 43–50. *In* E. Quagliariello and F. Palmieri (ed.), Molecular mechanisms of transport. Elsevier Science Publishers B.V., Amsterdam.
38. **Unemoto, T., H. Tokuda, and M. Hayashi.** 1990. Primary sodium pumps and their significance in bacterial energetics, p. 33–54. *In* T. A. Krulwich (ed.), The bacteria, vol. XII. Bacterial energetics. Academic Press, Inc., San Diego, Calif.
39. **Van Kessel, J. S., and J. B. Russell.** 1992. Energetics of arginine and lysine transport by whole cells and membrane vesicles of strain SR, a monensin-sensitive ruminal bacterium. Appl. Environ. Microbiol. **58:**969–975.
40. **Verhoogt, H. J. C., H. Smit, T. Abee, M. Gamper, A. J. M. Driessen, D. Haas, and W. N. Konings.** 1992. *arcD*, the first gene of the *arc* operon for anaerobic arginine catabolism in *Pseudomonas aeruginosa*, encodes an arginine-ornithine exchanger. J. Bacteriol. **174:**1568–1573.
41. **Walderhaugh, M. O., D. C. Dosch, and W. Epstein.** 1987. Potassium transport in bacteria, p. 85–130. *In* B. P. Rosen and S. Silver (ed.), Ion transport in prokaryotes. Academic Press, Inc., San Diego, Calif.