Sodium-stimulated transport of glutamate by *Thermus thermophilus* strain B

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Thermus thermophilus B is one of the many *Thermus* strains able to utilize glutamate as a sole source of carbon. Sodium is an obligate requirement for glutamate transport by washed whole cells, but the affinity for sodium ($K_m = 23$ mM) is low. At pH 7.6, uptake of glutamate is rapid in 50 mM-sodium at 65 °C and obeys saturation kinetics with an apparent K_m of 0.23 μ M-glutamate and a V_{max} of 12 nmol glutamate min⁻¹ (mg protein)⁻¹. The transport system is insensitive to osmotic shock and is specific for glutamate, with both the L- and D-isomers being transported. Uptake is very sensitive to inhibitors that collapse the membrane potential ($\Delta\psi$) or the chemical gradient of sodium ions (ΔpNa), but a transmembrane pH gradient (ΔpH) plays no role in the transport of glutamate. These results are therefore consistent with a membrane sodium/glutamate symport system.

Introduction

Thermus species are thermophilic, aerobic, Gram-negative heterotrophs. Yellow isolates are similar to the type strain (YT1) of *Thermus aquaticus* (Brock & Freeze, 1969), and grow optimally at approximately 70 °C. Red pigmented strains of *Thermus ruber* (Loginova & Egorova, 1975) have lower temperature optima close to 60 °C. Many strains of both types have been isolated from natural (Hudson *et al.*, 1989; Munster *et al.*, 1986; Pask-Hughes & Williams, 1977; Santos *et al.*, 1989) and man-made (Brock & Boylen, 1973; Pask-Hughes & Williams, 1975) thermal sources worldwide. Yellow pigmented *Thermus* isolates are heterogeneous, with several genospecies detected by DNA:DNA hybridization (Williams, 1989). One homology group corresponds to '*Thermus thermophilus*' (Oshima & Imahori, 1974). *Thermus* strain B (NCIB 11247), the subject of the present study, has a high DNA:DNA homology with strain HB8.

Studies of the physiology and growth of these organisms (Bergquist *et al.*, 1987; McKay *et al.*, 1982; Wiegel & Ljungdahl, 1984) have paid little attention to the transport of nutrients across the membrane. Some

active transport processes in Gram-negative bacteria are sensitive to osmotic shock, which disables transport by releasing the substrate-binding proteins from the periplasmic space, but causes no loss in viability. The protein that specifically binds a substrate, often with high affinity, may be isolated from the 'shock' fluid. This periplasmic binding protein, complexed with its substrate, interacts with translocation proteins that hydrolyse ATP at the cytoplasmic surface of the membrane (Ames, 1986). A second class of transport processes often have lower affinities for their substrates and are insensitive to osmotic shock. They are commonly energized by a proton (or other ion) motive force, and substrate uptake occurs in symport (cotransport) with the ion (Kaback, 1986, 1990; Maloy, 1990) in Gram-positive and Gram-negative micro-organisms. The proton motive force (pmf , $\Delta\mu_{H^+}$) is generated by proton pumping across a membrane that is otherwise impermeable to protons, and comprises an electrochemical gradient ($\Delta\psi$ positive to the outside) and a pH gradient (ΔpH acidic to the outside) that are related by the equation

$$pmf = \Delta\psi - Z\Delta pH \quad (Z = 67 \text{ at } 60^\circ\text{C})$$

Cells may form an energetically useful transmembrane gradient of other cations such as sodium, when $\Delta\mu_{Na^+}$ substitutes for $\Delta\mu_{H^+}$. Transport systems are known to depend on sodium in enteric bacteria (Cairney *et al.*, 1984; Chen & Russell, 1989; Fujimura *et al.*, 1983; Russell *et al.*, 1988), some thermophiles (De Vrij *et al.*, 1989, 1990; Speelmans *et al.*, 1989), and in alkaliphilic

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and marine micro-organisms for which sodium plays a special role (Dimroth, 1987; Maloy, 1990; Krulwich & Ivey, 1990). Sodium dependent transport systems may also involve special sodium extrusion systems. For alkaliphiles sodium/proton antiporters serve to acidify the cytosol and generate inwardly directed sodium motive force (Krulwich & Guffanti, 1989). Many halophiles have a respiration-dependent primary sodium pump that provides energy for substrate uptake (Unemoto & Hayashi, 1989; Unemoto *et al.*, 1990). Halotolerant bacteria have several mechanisms for the extrusion of sodium ions (De Vrij *et al.*, 1990). This paper, which is part of a study of the growth physiology of *Thermus* (Holtom, 1991), describes a high affinity glutamate transport system in *Thermus* strain B that is insensitive to osmotic shock and dependent on sodium ions.

Methods

Growth and maintenance. *Thermus* strain B was isolated in Iceland (Williams, 1975) and since maintained freeze-dried. In the short term, working stock suspensions were stored frozen. The growth medium (TYE) used for culturing *Thermus* contained 0.3% (w/v) tryptone and 0.1% (w/v) yeast extract with 10% (v/v) of $\times 10$ Castenholz mineral salts solution (Ramaley & Hixson, 1970). Cells were cultured at 60 °C in an orbital incubator and aerated by shaking at 200 r.p.m. The culture was stored on plates of TYE medium containing 2% (w/v) agar at 4 °C and subcultured every 2 weeks. Viable counts were performed using a 0.1 ml spread plate technique using sterile TYE broth as a diluent.

Glutamate uptake assays. Mid exponential phase cells, with an OD₆₀₀ of approximately 1.8, were harvested by centrifugation for 2 min at 7000 g in an MSE Microcentaur at room temperature. The cells were washed once in the assay buffer followed by centrifuging for 1 min at 7000 g at room temperature and resuspended at concentrations of 3–5 µg cell protein ml⁻¹. These dilute suspensions were necessary to investigate the high affinity glutamate transport system, and were either used immediately, or stored at 4 °C for 1–4 h before use. Such storage made no difference to the rate of glutamate transport. Aliquots of 9 ml of cell suspension were pre-incubated for 10 min at the assay temperature in a New Brunswick G76D shaking water-bath. The uptake of glutamate was usually initiated by addition of 1 ml 1 µM L-[U-¹⁴C]glutamate [270 mCi mmol⁻¹ (about 10 GBq mmol⁻¹), the highest specific activity available], to a final concentration of 0.1 µM. Samples (1 ml containing 0.025 µCi equivalent to 60000 d.p.m.) were taken at 15 s intervals and filtered rapidly through cellulose nitrate filters (Whatman), 0.45 µm pore size. The filters were then washed with 2 ml of cold sterile assay buffer and dried at 80 °C for 1 h. PCS scintillant (Amersham) was added (3.5 ml) to the dried filters and the radioactivity counted in an LKB 1215 liquid scintillation counter. Typical count rates for filters were about 300 c.p.m. against a background of 30 c.p.m. Where ionophores were used, these were added (concentrations in Table 1) to the incubation mixture 2 min before substrate addition.

Control bacterial suspensions were boiled for 15 min and used to test the non-specific binding of glutamate to the cells and filters. Boiled controls were used because in unboiled control suspensions glutamate transport occurred, even after several hours in the absence of an exogenous energy source. These control counts were subtracted from experimental results. Lines of best fit were calculated from data points

using linear regression analysis, and the kinetic constants were calculated using the EZ-FIT kinetics software package (Perella, 1988). The graphs provided are illustrative and were not used to calculate kinetic constants.

Osmotic shock and chloroform treatment. These treatments were used to attempt the selective release of periplasmic proteins (Ames *et al.*, 1984; Venegas *et al.*, 1980).

Treatment of protein and equilibrium dialysis. For glutamate binding dialysis tubing was boiled in 2% (w/v) sodium bicarbonate and in 1 mM-EDTA, stored in 1 mM-EDTA at 4 °C, and rinsed well in demineralized water. Cellular proteins (0.1–0.5 mg ml⁻¹) were reversibly denatured by dialysing against 100 vols 50 mM-Tris/HCl, pH 7.8, 5 mM-EDTA, 5 mM-DTT and 6 M-guanidine hydrochloride, and re-natured by dialysis against 3 $\times$ 100 vols 50 mM-Tris/HCl, pH 7.8, 5 mM-EDTA and 2 mM-DTT. Samples of protein were assayed for glutamate binding by equilibrium dialysis in a series of dialysis bags immersed in 5 ml bottles each containing 25 pmol L-[U-¹⁴C]glutamic acid in 2 ml of the same buffer. Some dialyses also contained 50 mM-NaCl. After overnight incubation at 60 °C, 100 µl aliquots from inside and outside each dialysis bag were mixed with 3.5 ml of PCS scintillant and counted in an LKB 1215 liquid scintillation counter.

Protein estimation. Protein concentrations were measured by the Coomassie dye binding method (Bradford, 1976) using Pierce reagent.

Materials. L-[U-¹⁴C]Glutamic acid was from Amersham and [methyl-³H]triphenylphosphonium iodide was from New England Nuclear. All other chemicals were of the highest purity obtainable.

Results

Cation specificity

No radioactivity above background was detected in cells and therefore no glutamate uptake took place in *Thermus* strain B in 50 mM-potassium phosphate buffer at pH 7.6 and 55 °C, unless sodium ions were added (Fig. 1). Rates of glutamate uptake at 55 °C by cells stored at 4 °C in

Fig. 1. Effect of 50 mM-sodium (○) or -lithium (●) chloride or no addition (□) on the transport of glutamate by *Thermus thermophilus* strain B at pH 7.6 and 55 °C in 50 mM-potassium phosphate buffer.

Fig. 2. Effect of sodium concentration on the rate of glutamate transport by *Thermus thermophilus* strain B in 50 mM-potassium phosphate at pH 7.6 and 65 °C. A Hill plot of the same data is inset. In triplicate experiments the extremes were $\pm$ the following per cent of the means; 10 mM, 8.5%; 20 mM, 3.6%; 40 mM, 1.8%; 60 mM, 4.6%; 80 mM, 6.5%; and 100 mM 7.6%.

Fig. 3. Effect of temperature (a at pH 7.6) and pH (b at 65 °C) on the initial rate of glutamate uptake by *Thermus thermophilus* strain B in 50 mM-phosphate buffer containing 50 mM-sodium chloride. The mean and range of three determinations was 82 (81–83) at 65 °C and 45 (40–52) at 55 °C (a), and 60 (58–62) at pH 7.0 and 39 (36–41) at pH 8.25 (b).

this buffer were constant for several hours. No glutamate transport occurred when 50 mM-lithium replaced sodium. Similar results with the cationic buffers Tris, TES and HEPES in place of potassium phosphate showed that glutamate transport did not require potassium.

Fig. 4. Effect of increasing glutamate concentration on the rate of glutamate uptake by *Thermus thermophilus* strain B at 65 °C in 50 mM-phosphate buffer pH 7.6 and 50 mM-sodium chloride. Results are means and ranges for triplicate estimations. The K_m and V_{max} were calculated by EZ-FIT, not from this graph.

Cells suspended in 50 mM-potassium phosphate buffer pH 7.6 were diluted into similar buffers between pH 6.0 and 8.0 and containing 0.1 μ M radiolabelled glutamate at the highest possible specific activity at 55 °C. The different degrees of Δ pH so generated did not drive glutamate uptake unless sodium was added, indicating that Δ pH plays no role under these conditions. Increasing the sodium concentration in the incubation medium at pH 7.6 caused a progressive increase in the rate of glutamate uptake (Fig. 2). The apparent K_m for sodium was approximately 23 mM, which is less than half the sodium concentration routinely used in buffers in these experiments. The Hill plot for this data (Fig. 2 inset) had a slope of one.

Kinetics of glutamate transport

The optimum temperature and pH for glutamate uptake in strain B were 75 °C and pH 7.6 respectively (Fig. 3). At 75 °C, however, the assay buffer evaporated rapidly and assays were therefore routinely performed at 65 °C at which temperature the rate was 90–95% of that at 75 °C. Under these conditions, the kinetic parameters of transport were determined from initial rates of uptake in 50 mM-potassium phosphate buffer and 50 mM-sodium, at glutamate concentrations between 0.025 μ M and 1 μ M at high specific activities (Fig. 4). The apparent K_m for transport was 0.23 μ M-glutamate and the V_{max} 12 nmol glutamate min^{-1} (mg protein) $^{-1}$.

Effect of osmotic shock

An osmotic shock procedure (Venegas *et al.*, 1980) was carried out three times on cells of *Thermus* strain B capable of active transport. The first shock resulted in no

loss of viability or transport activity. The second and third shocks reduced viability by 63% and over 99%, and reduced glutamate transport by 5% and 71% respectively. No glutamate binding protein was detected by equilibrium dialysis of any of the three shock fluids, whether the proteins were reversibly denatured or untreated, and in the presence or absence of NaCl. The attempted release of periplasmic binding protein using chloroform (Ames *et al.*, 1984) resulted in immediate and complete loss of viability and transport activity, but again no glutamate binding proteins were detected by equilibrium dialysis.

Effect of inhibitors

Ionophores that collapse the proton motive force, carbonyl cyanide chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol (2,4-DNP) were very inhibitory to glutamate uptake in *Thermus* strain B (Table 1) in 50 mM-phosphate buffer pH 7.6 containing 50 mM-sodium ions at 65 °C. This might be due to a collapse in $\Delta\psi$, ΔpH or both, but other evidence cited above suggests that ΔpH is unimportant. The sensitivity of glutamate uptake to thiocyanate and valinomycin (Table 1), both of which specifically collapse $\Delta\psi$ under the assay conditions, demonstrates the importance of $\Delta\psi$ and implies that glutamate uptake is electrogenic.

These observations, and the obligate requirement of sodium for glutamate transport, imply that transport may be energized by a sodium motive force, and therefore a chemical gradient of sodium ions (ΔpNa) would be

Table 1. Effect of protonophores, collapsers of $\Delta\psi$, monensin and nigericin on glutamate transport by *Thermus* strain B

The concentrations of inhibitor cited are the minimum concentrations required to bring about the described degree of inhibition. The inhibitors were added 2 min before the addition of L-[U- ^{14}C]glutamate to a final concentration of 0.1 μM , in buffer containing 50 mM-sodium ions at pH 7.6 and 65 °C.

Compound	Concn	Buffer (50 mM)	Inhibition (%)
Control		Phosphate	0
2,4-DNP	5 mM	Phosphate	91
CCCP	50 μM	Phosphate	96
Valinomycin	0.7 nM	Phosphate	99
NaSCN	10 mM	Phosphate	97
Control		Phosphate	0
Monensin	0.03 μM	Phosphate	98
Nigericin	0.4 μM	Phosphate	94
Control		Tris	0
Monensin	0.03 μM	Tris	99
Nigericin	1 μM	Tris	95

expected to be significant. This gradient would be collapsed by the sodium/proton antiporter monensin, which preferentially exchanges one sodium ion for one proton and has no effect on $\Delta\psi$. Monensin inhibited the transport of glutamate by *Thermus* in both Tris and phosphate buffers (Table 1). Nigericin, a non-electrogenic antiporter that preferentially exchanges protons for potassium (or sodium with decreased affinity) was also inhibitory and mimicked monensin in Tris buffer without potassium, but monensin was 30 times as effective as nigericin. In the presence of potassium the nigericin concentration required to produce a similar degree of inhibition was halved. Under these conditions protons are exchanged for potassium ions. As neither potassium ions nor ΔpH is important for glutamate transport internal cellular pH may be significant.

Specificity

The specificity of the transport system was assessed from the extent of inhibition of L-[U- ^{14}C]glutamate uptake by a 30-fold excess of other amino acids (Table 2). Twenty of the compounds tested inhibited glutamate transport less than 18%. Only glutamine, leucine and valine inhibited more than the variation in control values. Increase in concentration of these three amino acids to 300 $\times$ the L-glutamate concentration resulted in no further inhibition. The presence of either α -oxoglutarate or L- α -aminopentanoic acid also caused no inhibition, indicating that an amino and two carboxylic acid groups are essential structural features of an inhibitor. The transport of radiolabelled L-glutamate was reduced to

Table 2. Effect of amino acids on the uptake of glutamate by *Thermus* strain B

Percentage inhibition of the uptake of L-[U- ^{14}C]glutamate (0.1 μM) by the presence of 3 μM unlabelled test substance. L-Serine, L-alanine and L-histidine caused 2–4% inhibition; L-glycine, L-proline, L-methionine, L-tryptophan, L-phenylalanine, L-tyrosine, L-cysteine, L-lysine, L-threonine, L-arginine and 2-oxoglutarate all gave no inhibition.

Test substance	Reduction in uptake of L-glutamate (%)
L-Glutamate	89
D-Glutamate	87
L-Glutamine	18
L-Leucine	18
L-Valine	13
L- α -Aminobutyrate	7
L-Aspartate	6
L-Asparagine	6
L-Isoleucine	6
L- α -Aminopentanoate	5

a similar extent by both unlabelled isomers. Therefore this system transports both the L- and D-isomers of glutamate.

Discussion

Many strains of the genus *Thermus* are able to utilize glutamate as the sole source of carbon (Hudson *et al.*, 1989; Munster *et al.*, 1986; Pask-Hughes & Williams, 1977). Transport of glutamate was studied with whole cells in this first study of *Thermus*. Uptake is rapid at pH 7.6 and increases with temperature up to 75 °C, but is negligible at 83 °C. This correlates well with the maximum temperature of the spring from which the strain was isolated (82 °C; Williams, 1975) and indicates that destabilization of the membrane may limit growth at high temperatures. The transport system described here is highly specific for glutamate of either isomer. Uptake of the L-isomer occurs with high affinity ($K_m = 0.23 \mu\text{M}$ -glutamate) and a maximum velocity of around 12 nmol glutamate min^{-1} (mg protein) $^{-1}$. Osmotic shock has no effect on transport which, in Gram-negative micro-organisms, is commonly associated with a periplasmic binding protein (Ames, 1986) that is released by such shocks.

Uptake is abolished by heating and by chemical inhibitors. This precludes the possibility of transport by passive diffusion. Sodium is obligatory for transport at pH 7.6 and no counts above background are taken up if sodium is omitted. The artificial imposition of a ΔpH at pH 6.0 is insufficient to drive transport in the absence of sodium, and does not stimulate transport in the presence of sodium. The *Thermus* transport system ($K_m = 23 \text{ mM}$) resembles that of *Streptococcus bovis* in its low affinity for sodium ($K_m = 30 \text{ mM}$), which prevented the direct demonstration of simultaneous sodium and amino acid uptake (Russell *et al.*, 1988). Direct sodium/glutamate cotransport was not detectable using either sodium ion selective electrodes or atomic absorption spectroscopy. These techniques are not sufficiently sensitive to detect changes of nmol quantities of ion against a background of 50 mM-sodium. The use of ^{22}Na was discounted because the specific activities available were not high enough to estimate sodium uptake at low sodium concentrations. In hot springs in Yellowstone National Park (Brock, 1978) and Iceland (Stainthorpe, 1986) concentrations of sodium are 2–20 mM so the low affinity is surprising and means that in the natural environment such a system may operate sub-optimally.

Glutamate uptake is sensitive to collapse of both the electrochemical gradient ($\Delta\psi$) and the chemical gradient of sodium ions (ΔpNa), but proceeds following the artificial imposition of ΔpNa and is inhibited by compounds that collapse it. Furthermore, transport of

glutamate is inactive at pH 6.0 (when the proton motive force comprises a large ΔpH with little $\Delta\psi$) although the strain can grow at this pH on complex media (Holtom, 1991). This provides more evidence that ΔpH plays no role and that $\Delta\psi$ is essential. The dependence of transport on $\Delta\psi$ implies that uptake is driven electrogenically, and predicts a net movement of positive charge. Glutamic acid has one net negative charge throughout the physiological pH range for transport, and therefore a net movement of positive charge requires at least two cations to be transported in symport with one glutamate. Despite this predicted stoichiometry, the effect of sodium ion concentrations upon transport obeys Michaelis–Menten first order kinetics, and a Hill plot of the data has a slope of one (Fig. 2). However, these results reflect overall transport activity and under such circumstances, the stoichiometry cannot be estimated.

A role for internal cell pH in the control of glutamate transport is suggested by the results with the ionophore nigericin. The internal pH affects some transport systems in other bacteria, but has not been reported for other sodium/solute symporters (Abee *et al.* 1989; Noji *et al.*, 1988). This effect is most likely due to the physiological consequences of altering the pH rather than to the alteration of ΔpH .

It has been suggested that the involvement of sodium in bacterial bioenergetics and membrane transport is relatively recent in evolution, with protons being the primitive currency for membrane energy transduction (Dimroth, 1987; Maloy, 1990). This argument suggests that sodium coupled symporters with differing cation specificities may represent stages in the evolution from proton to sodium driven energetics. However, on the basis of 16S ribosomal RNA sequencing *T. thermophilus* is a particularly ancient organism, being on the deepest branch on the eubacterial phylogenetic tree after the genus *Thermotoga* (Hartmann *et al.*, 1989). Since glutamate transport requires, and is driven by, sodium in *Thermus* strain B, it therefore seems that a role for sodium in bioenergetics may have evolved earlier than has been supposed.

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