TRANSPORT OF L-GLUTAMATE AND L-ASPARTATE BY MEMBRANE VESICLES OF *BACILLUS SUBTILIS* W 23

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1. Introduction

Isolated membrane vesicles (kabackosomes) [1] permit the investigation of the biochemical properties of transport processes in the cytoplasmic membrane. We have selected *Bacillus subtilis* for the amino acid transport studies in micro-organisms because kabackosomes of gram-positive organisms, that do not possess periplasmic proteins, most likely contain the complete cellular transport system. In a previous publication [2] it was shown that in *B. subtilis* the amino acids can be ordered in nine groups according to their effect on each other's transport. The amino acids of each group mutually inhibit each other's transport but not the transport of other amino acids. These observations indicate that amino acid transport is catalyzed by at least 9 distinct carrier proteins, each of which is specific for a group of structurally related amino acids. However, the data do not exclude the possibility that the transport of amino acids within a specific group is catalyzed by several carriers with different affinities. The purpose of this investigation was to examine the number of carriers for one group of amino acids, *viz.* L-glutamate and L-aspartate. Several studies of dicarboxylic amino acid transport systems in whole cells have been reported for *E. coli* [3–5] and *Streptococcus faecalis* [6, 7] and a dicarboxylic acid transport system in *B. subtilis* was reported while this work was in progress [8].

Both L-aspartate and L-glutamate are transported at a high rate by *B. subtilis* kabackosomes, which facilitates kinetic investigation. The transport of both amino acids is performed by a highly specific carrier protein, which has no affinity for dicarboxylic acid anions such as succinate, malate and fumarate.

2. Methods

Bacillus subtilis W 23 was grown at 37° in a medium containing 0.8% Bacto-Tryptone (Difco Laboratories, Detroit, Michigan, USA) and 0.5% NaCl. Logarithmically growing cells were harvested when the medium had reached an absorbancy of 1.0–1.5 at 660 nm (A_{660}) and washed twice in 0.1 M K-phosphate, pH 7.3. Kabackosomes were isolated by a modification of a previously published procedure* [2, 9].

Uptake experiments were performed as described previously [2, 9, 10]. The reaction was terminated by the addition of 2 ml 0.1 M LiCl and the suspensions were then filtered on Schleicher and Schüll BA 85 (pore size 0.45 μm, 25 mm diameter). The filters were washed with 2 ml 0.1 M LiCl, dried in counting vials for 20 min at 105°. After the addition of 10 ml toluene, supplemented with 5 g/l 2,5-diphenyloxazole, the radioactivity was measured in a scintillation counter.

The ^{14}C compounds (Amersham, England) used were of the highest specific activity available: L-glutamate 270 mCi/mmole, uniformly labeled, L-aspartate 227 mCi/mmole, uniformly labeled and [1,4-^{14}C] succinate 20.4 mCi/mmole. They were stored at −10° at concentrations of 10^{-4}–10^{-5} M.

* W.N. Konings, A. Bisschop and M.C.C. Daatselaar, manuscript in preparation.

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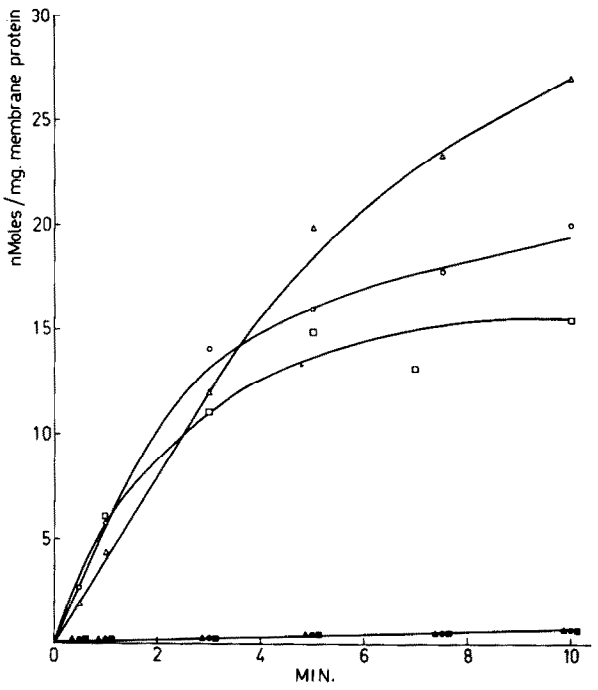

Fig. Time course of the uptake of L-[^{14}C]glutamate, L-[^{14}C]aspartate and [^{14}C]succinate by *B. subtilis* W 23 kabackosomes in the presence of 40 mM Na-ascorbate and 100 μM PMS (open symbols) and without an electron donor (closed symbols). The incubation mixture (0.1 ml) contained 0.043 mg membrane protein. (○—○—○) L-[^{14}C]glutamate (9.3 μM); (△—△—△) L-[^{14}C]aspartate (22 μM); (□—□—□) [^{14}C]succinate (122.5 μM).

3. Results

Kabackosomes of *B. subtilis* W 23 transport L-glutamate, L-aspartate and succinate* at a high rate in the presence of ascorbate-phenazine methosulfate (PMS) as the electron donor [2, 9, 10], but they hardly accumulate these compounds in the absence of an energy source (fig. 1). Their concentrating power is remarkable: the steady state internal concentration is about 6.7 mM for L-glu, 9 mM for L-asp, and 5 mM for succinate (calculated from a total vesicle volume of 3 μl per mg membrane protein [2]), which is a concentrating power of 725, 400 and 41 fold, respectively. The transport activity of these membrane preparations (V_{max} values in table 1) is comparable to that of logarithmically growing whole cells: the V_{max} for L-glutamate uptake in whole cells is about 90 nmoles/min/mg membrane protein (calculated with the assumption that 15% of the total protein is membrane protein [11]). Taking into account losses during the isolation procedure the figures strongly indicate that the mechanism for transport in these vesicles is identical to that of intact cells.

* A. Matin and W.N. Konings, manuscript in preparation.

Table 1
Kinetic constants for transport of ^{14}C-compounds in kabackosomes of *B. subtilis* W 23.

Compounds	K_m (μM)	K_i (μM)	V_{max} (nmoles/min/mg protein)
L-glutamate	13	13*	38
L-aspartate	27	25†	17
Succinate	4.5	–	6.1

* Determined by the inhibition of the initial rate of transport of L-aspartate (see fig. 3b).
† Determined by the inhibition of the initial rate of transport of L-glutamate (see fig. 3a).
Data were obtained as described in Methods and in fig. 3.

The number of carrier proteins involved in the active transport of L-glu and L-asp was investigated. If both amino acids are transported by the same carrier protein(s), the addition of either amino acid to kabackosomes that have concentrated the other amino acid will cause counterflow, i.e. the added amino acid is taken up while simultaneously a release of the concentrated amino acid occurs. Indeed, as is shown in fig. 2, the addition of L-asp to kabackosomes, which have concentrated L-glu to the steady state level, results in a rapid counterflow of L-glu. The efflux of L-glu is about equal to the efflux observed when unlabeled L-glu is added. Likewise, an efflux of L-aspartate occurs when unlabeled L-glu is added to membrane vesicles which have concentrated L-asp. The efflux of the accumulated amino acid is almost complete, which indicates that the carrier protein(s) involved in the transport of L-asp and L-glu does have affinity for both amino acids.

The effect of L-glu on the initial rate of uptake of L-asp, and vice versa, was studied. The kinetic experiments shown in fig. 3 reveal that both amino acids inhibit each other's transport competitively

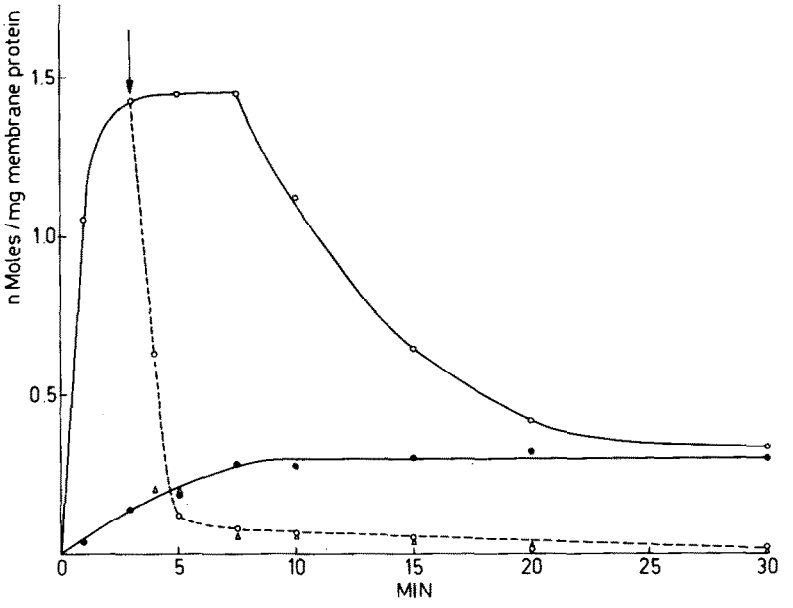

Fig. 2. Uptake of L-[^{14}C]glutamate by *B. subtilis* W 23 kabackosomes (isolated as described in [2]). At the time indicated by the arrow, 1 mM unlabeled L-glutamate or 1 mM unlabeled L-aspartate was added. The concentration of L-[^{14}C]glutamate was 9.3 μM; the incubation mixture (0.1 ml) contained 0.06 mg membrane protein. The uptake measurements were performed as described in Methods. (○—○—○).Uptake with 40 mM Na-ascorbate and 100 μM PMS as electron donor; (●—●—●) uptake without the addition of an electron donor; (○ - - ○ - - ○) counterflow by the addition of 1 mM L-aspartate; (△ - - △ - - △) counterflow by the addition of 1 mM L-glutamate.

in the concentration range 2 to 40 μM. Although these observations suggest that in this concentration range there is only one carrier for both amino acids, they do not rule out the possibility that there are carriers specific for each amino acid and the inhibiting amino acid acts by blocking the binding site for the transported amino acid (the linearity of the Lineweaver-Burk plot makes the involvement of more than one carrier for either amino acid unlikely). This possibility is ruled out, however, because the affinity constants for the transport of amino acids (K_m) are equal to the affinity constant for the inhibitory action (K_i) (table 1). Thus the transport of L-glu and L-asp in kabackosomes of *B. subtilis* is mediated by only one carrier protein.

The carrier is highly specific for the acidic amino acids as was shown by competition experiments with other amino acids. The concentration of L-asp and L-glu, respectively, was about the K_m value; the competing amino acid was added at final concentration of four times the K_m value [2] (L-asparagine and L-glutamine were not used since they are deamidated by the membrane preparation [2]). None of the amino acids inhibited the initial rate of L-glu and L-asp uptake more than 25%, which agrees with observations made in *B. subtilis* 60015 [2].

In addition, the effect on the initial rates of uptake by a wide variety of mono-, di- and tricarboxylic acids at 1 mM concentration was studied (table 2). Only cysteic acid strongly inhibits the uptake of L-glu and L-asp. Of special interest is the observation that none of the dicarboxylic acid anions (succinate, fumarate, malate) markedly affected the transport of either amino acid.

The experiments described were done in the concentration range up to 40 μM, so they do not exclude the possibility that an additional carrier with a low affinity for both amino acids exists. A possible candidate might be the transport system for dicarboxylic acids. Kabackosomes actively transport succinate in the presence of an electron donor* (fig. 1

* A. Matina and W.N. Konings, manuscript in preparation.

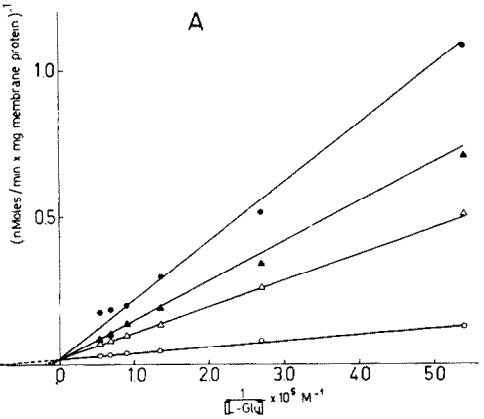

Fig. 3a. Inhibition of L-[^{14}C] glutamate by L-aspartate and of L-[^{14}] aspartate by L-glutamate. The initial rates of uptake were determined after 1 min incubation with 0.043 mg membrane protein as described in Methods. L-[^{14}C] glutamate transport; concentration of L-aspartate used: (●—●—●) 160 μM; (▲—▲—▲) 80 μM; (△—△—△) 40 μM; (○—○—○) 0 μM.

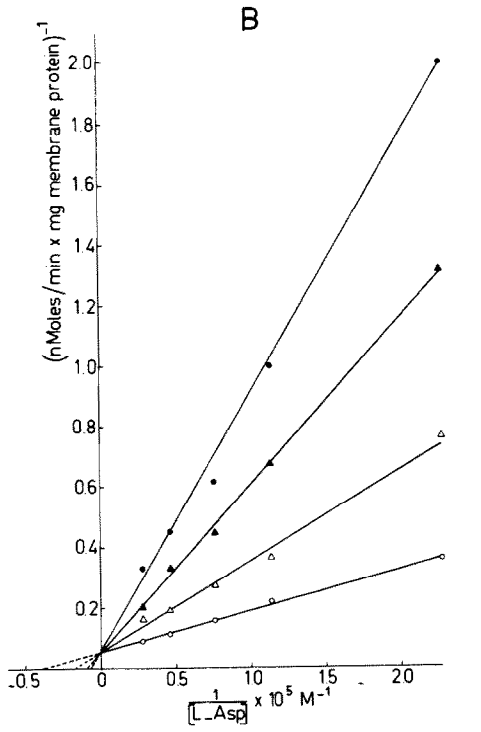

Fig. 3b. Inhibition of L-[^{14}C] glutamate by L-aspartate and of L-[^{14}C] aspartate by L-glutamate. The initial rates of uptake were determined after 1 min incubation with 0.043 mg membrane protein as described in Methods. L-[^{14}C] aspartate transport; concentrations of L-glutamate used: (●—●—●) 100 μM; (▲—▲—▲) 50 μM; (△—△—△) 25 μM; (○—○—○) 0 μM.

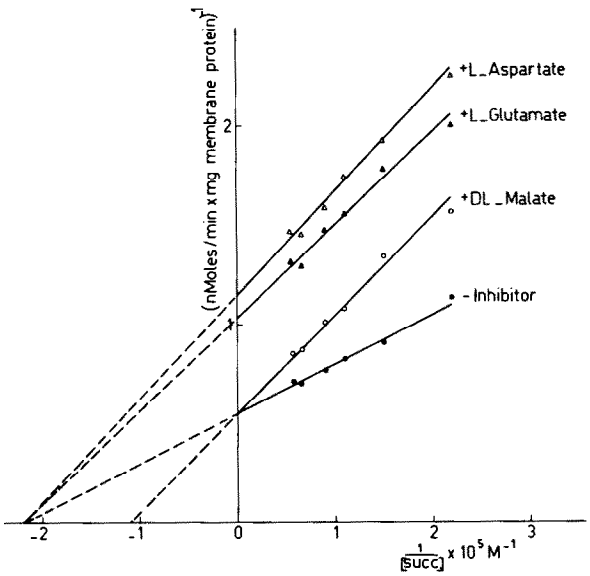

Fig. 4. Inhibition of [^{14}C] succinate transport by L-aspartate (50 μM), L-glutamate (26 μM) and D,L-malate (10 μM). The experiments were performed as described in fig. 3 and in Methods.

The effect of mono-, di- and tricarboxylic acids on the initial rate of transport of succinate is shown in table 2. The dicarboxylic acid anions fumarate, malate and α-ketoglutarate markedly inhibit the transport of succinate. L-aspartate and L-glutamate, also, strongly inhibit succinate uptake. However, competition experiments (fig. 4) show that they inhibit the transport of succinate non-competitively (K_i's are in the order of 10^{-5} M), while D,L-malate inhibits succinate transport competitively (K_i about 10^{-5} M). This rules out the possibility that the carrier for succinate may transport L-glu and L-asp with low affinity.

4. Discussion

The transport of L-glutamate and L-aspartate occurs via a highly specific carrier protein. The following lines of evidence support this contention:

1) Transport of one amino acid causes counterflow of the previously accumulated other amino acid. Sufficiently high concentrations of the external amino acid cause an efflux of more than 95% of the accumulated amino acid.

Table 2
Percentage inhibition of initial rate of transport.

Inhibitors (1 mM)	^{14}C-compounds transported		
	L-glutamate	L-aspartate	Succinate
L-glutamate	97	95	81
L-aspartate	92	95	87
Succinate	8	2	97
D,L-malate	8	26	97
Fumarate	13	26	99
α-Ketoglutarate	0	23	41
Cysteic acid	70	72	29
Oxalate	15	30	n.d.
Malonate	4	24	11
Citrate	14	20	16
Pyruvate	12	24	24
D-lactate	3	3	21
L-lactate	0	4	21
Oxamate	20	5	10
Glycolate	16	23	21

n.d. = not determined.

The initial rate of uptake was measured in kabackosomes (0.86 mg membrane protein/ml) at 25°. After the addition of 1 mM unlabeled inhibitor, 40 mM ascorbate pH 6.6, 100 μM PMS and the ^{14}C-compounds were added in rapid succession. The mixture was incubated for 1 min. Percentages inhibition were calculated with respect to uptake of the ^{14}C-compounds in the absence of inhibitors. The concentrations of the ^{14}C-compounds used were: L-glutamate: 9.3 μM; L-aspartate: 11.0 μM; succinate 12.25 μM.

2) Both amino acids inhibit each other's transport competitively. The affinity constants in the transport process and in the inhibition are equal.
3) The transport of both amino acids is only slightly inhibited by the presence of high concentrations of other mono-, di- and tricarboxylic acids or by the presence of other amino acids (except cysteic acid).
4) Neither amino acid is transported by a dicarboxylic acid carrier protein. Ghei et al. [8] observed in malate grown cells of *B. subtilis* 168 that 1 mM L-aspartate inhibits the transport of succinate by 22% and of malate not at all. In these cells succinate inhibits its own transport by 89% but the transport of malate by only 37%. In contrast, in citrate grown cells, succinate inhibits malate transport by 78%.

These observations, combined with the results in kabackosomes described above, strongly suggest the existence of a second (inducible) transport system for dicarboxylic acids (but not for dicarboxylic amino acids) which has affinity for malate mainly. The low inhibition of succinate transport by L-aspartate observed by Ghei et al. [8] might be due to the non-competitive inhibition of the above described carrier for succinate.

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