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Active Transport of D-Alanine and Related Amino Acids by Whole Cells of *Bacillus subtilis*

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Whole cells of *Bacillus subtilis* transported D-alanine and L-alanine by two different systems. The high-affinity system (K_m of 1 μM and V_{max} of 0.6 to 0.8 nmol/min per mg of protein) was specific for the two stereoisomers of alanine. The low-affinity system (K_m of 10 μM for L-alanine and 20 μM for D-alanine and glycine) had a V_{max} of 5 to 12 nmol/min per mg of protein. This system transported glycine, D-cycloserine, and D-serine, in addition to D- and L-alanine. Azide inhibited the uptake of these amino acids and caused the efflux of D-alanine from preloaded cells. These data suggest that transport of these amino acids is energized by the electron transport chain.

D-Alanine is an integral component of the cell wall polymers, peptidoglycan and teichoic acid, and of the membrane polymer, lipoteichoic acid, in *Bacillus subtilis* 168 (16). In searching for mutants in cell wall biosynthesis, we constructed a mutant deficient in D,L-alanine racemase (EC 5.1.1.1) (3). This enzyme catalyzes the racemization of L-alanine to D-alanine (8), which is the first step in the incorporation of D-alanine into peptidoglycan. The D-alanine is condensed into a dimer, D-alanyl-D-alanine, by the action of the ligase enzyme (D-alanine:D-alanine ligase [adenosine-5'-diphosphate-forming] [EC 6.3.2.4]), and this dimer is transferred to uridine 5'-diphosphate-muramyl tripeptide to form uridine 5'-diphosphate-muramyl pentapeptide, the building block for peptidoglycan (16). The enzymatic reactions involved in the incorporation of D-alanine into wall and membrane teichoic acid are not well understood (11, 17). Since D-alanine is a necessary component of wall and membrane polymers, it may serve a role as a regulatory element in the coordination of wall and membrane synthesis. For this reason, we have begun a study of the metabolism and function of D-alanine in *B. subtilis*. The first step is the study of the mode of transport of D-alanine into the cell.

D-Alanine transport has been studied in whole cells of *Escherichia coli* (2, 5, 15, 18, 19, 22, 23), *Lactobacillus casei* (9), and *Streptococcus faecalis* (14), as well as in membrane vesicles of *E. coli* (12), *Staphylococcus aureus* (20, 21), and *B. subtilis* (6). Whereas it is found that D-alanine, L-alanine, and glycine are all carried by one system in *E. coli* membrane vesicles (12), it has been observed that there are multiple systems in whole cells of *E. coli*. There also seems to be some disagreement about the nature of the system(s) that carry these three amino acids. Schwartz et al. (19) reported that a D-serine-resistant mutant of *E. coli* was defective in D-serine, L-alanine, and glycine transport, implying that these amino acids are transported by the same system. Kessel and Lubin (5) determined that glycine, L-alanine, D-alanine, D-serine, and D-cycloserine are all transported by the same system, which was not involved in the transport of L-serine. However, Piperno and Oxender (15) reported the presence of a common transport system for glycine, alanine, and serine that was not stereospecific, transporting both D- and L-isomers of the latter two amino acids. Wargel et al. (22, 23) subsequently reported the existence of two related transport systems responsible for the accumulation of D-alanine, glycine, and D-cycloserine, and a third separate system for the transport of L-alanine. The most recent work on the transport of these amino acids in *E. coli* (2, 18) described the existence of two transport systems, one specific for glycine, D-alanine, D-cycloserine, and D-serine that may also be capable of transporting L-alanine, and a second system specific for L-alanine which may also transport L-serine. In *L. casei* (9), two transport systems are present, one specific for glycine, and the other specific for both stereoisomers of alanine. *S. faecalis* (14) contains a single transport system for glycine, L-alanine, and D-alanine.

Thus, in whole cells there is no constant pattern for transport of the stereoisomers of alanine and serine.

Membrane vesicles of *E. coli* (12), *S. aureus* (20, 21), and *B. subtilis* (6) contain only one system which transports glycine, L-alanine, D-alanine, and D-serine, but not L-serine. With the exception of the histidine and the leucine-isoleucine-valine transport systems of *E. coli* (12), the amino acid transport systems seen in membrane vesicles contain only one K_m value for the amino acid transported, although transport systems are known to occur in whole cells that have multiple saturable components with differing affinities for the amino acid transported (1, 4, 22, 23). Therefore, it has been postulated (12) that some of the components necessary for transport may be lost during the preparation of the membrane vesicles.

We would like to present evidence for the existence of two transport systems in whole cells of *B. subtilis* 168. The first, a high-affinity system, accumulates D- and L-alanine, and the second, a low-affinity system, accumulates D- and L-alanine, glycine, D-cycloserine, and D-serine.

MATERIALS AND METHODS

Strains. *B. subtilis* 168, carrying *dal-1*, *hisAl*, *leuA*, and *metB10* (3), was used throughout this study. Strains carrying *dal-1* are blocked in D,L-alanine racemase; therefore, this strain requires D-alanine for growth.

Growth conditions and preparation of cells for transport. Cells were grown at 37 C in Spizizen minimal salts medium (24) supplemented with 22 mM glucose, 0.5% casein hydrolysate, and 0.5 mM D-alanine. Turbidity was determined with a Klett-Summerson colorimeter at 620 nm. The medium was inoculated from an overnight culture of cells to give an initial turbidity reading of 10 Klett units. The cells were grown to late exponential phase (about 150 Klett units), harvested by membrane filtration (Millipore Corp., 0.45-μm pore size, 45-mm diameter), washed with Spizizen minimal salts, and suspended in Spizizen minimal salts supplemented with 0.5 mM D-alanine. The addition of D-alanine was necessary to maintain transport and membrane stability (V. L. Clark and F. E. Young, manuscript in preparation). Portions (10 ml) of cells were removed at time periods ranging from 5 to 30 min after resuspension, filtered, washed, and suspended in 30 ml of Spizizen minimal salts without D-alanine. These cells were incubated at 37 C for 5 min and used in all transport studies.

Measurement of transport. Uptake was initiated by the addition of 2.0 ml of cells to 0.5 ml of minimal salts containing the ^{14}C-labeled amino acid to be transported plus transport inhibitors when appropriate. The cells were incubated at 37 C for 30 s or 15 min, depending upon whether initial rate or net transport, respectively, was measured. Azide sensitivity was determined with cells preincubated for 5 min at 37 C in Spizizen minimal salts plus 10 mM sodium azide. Transport was terminated by membrane filtration (Millipore Corp., 0.45-μM pore size, 24-mm diameter). Cells were washed once with 2 ml of Spizizen minimal salts, and the filters were placed in scintillation vials and dried overnight at 45 C; 5 ml of scintillation fluid (15.2 g of Omnifluor per 3.8 liters of toluene) was added, and the vials were counted in a Beckman LS 230 liquid scintillation spectrometer. The protein concentration was determined by the method of Lowry et al. (13) for trichloroacetic acid precipitates, with bovine serum albumin as a standard.

Materials. The D-[^{14}C]alanine was purchased from Amersham/Searle Corp. and had a specific activity of 36 mCi/mmol. L-[^{14}C]alanine (specific activity of 153 mCi/mmol) and [^{14}C]glycine (specific activity of 92 mCi/mmol) were obtained from New England Nuclear, as was the Omnifluor used in the preparation of the scintillation fluid. The unlabeled amino acids, D-alanine, D-cycloserine, and L-alanine, and the sodium azide were purchased from Sigma Chemical Co. Glycine was bought from Fisher Scientific Co., D-alanyl-D-alanine from Cyclo Chemical, and D-serine from General Biochemicals. All other chemicals were of reagent grade purity.

RESULTS

The rate of D-alanine uptake by whole cells of *B. subtilis* was constant for the first 2 min (data not shown). To insure that the initial rate was being measured, cells were allowed to accumulate amino acid for only 30 s. Kinetic analysis of the substrate saturation characteristics for the initial rate of uptake of D-alanine (Fig. 1) revealed the presence of two apparent K_m values for D-alanine, 1 μM and 20 μM. Identical K_m and V_{max} values were obtained when uptake occurred in the presence of 0.4% glucose (data not shown). The deviation from linearity in the Lineweaver-Burk (10) plots at high concentrations of D-alanine was due to azide insensitive uptake and was eliminated by correcting for this by subtraction of the uptake that occurs in the presence of azide from the uptake that occurs in the absence of azide.

If net transport was measured by determining the amount of D-alanine incorporated after 15 min of incubation, when influx and efflux were in equilibrium, only one apparent K_m value for D-alanine was observed (Fig. 2). The K_m value of 20 μM D-alanine corresponded to the value obtained for the higher K_m system for initial uptake seen in Fig. 1. The V_{max} of 100 nmol per 15 min per mg of protein represented the maximal accumulation of D-alanine in the pool within the cell.

We also investigated the initial rate of transport of L-alanine and glycine. Figures 3 and 4

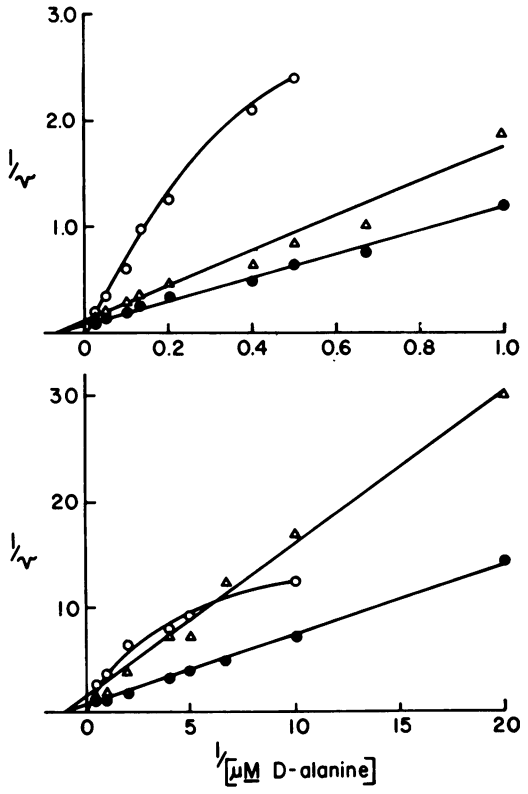

FIG. 1. *Lineweaver-Burk plots of* D-*alanine uptake. Velocity* (v) *is expressed as nmol/min per mg of protein. Symbols:* ●, *the observed rate of uptake;* ○, *the rate of uptake that occurs in the presence of 10 mM sodium azide;* △, *the rate of uptake after correction of the observed rate for the azide-insensitive uptake.*

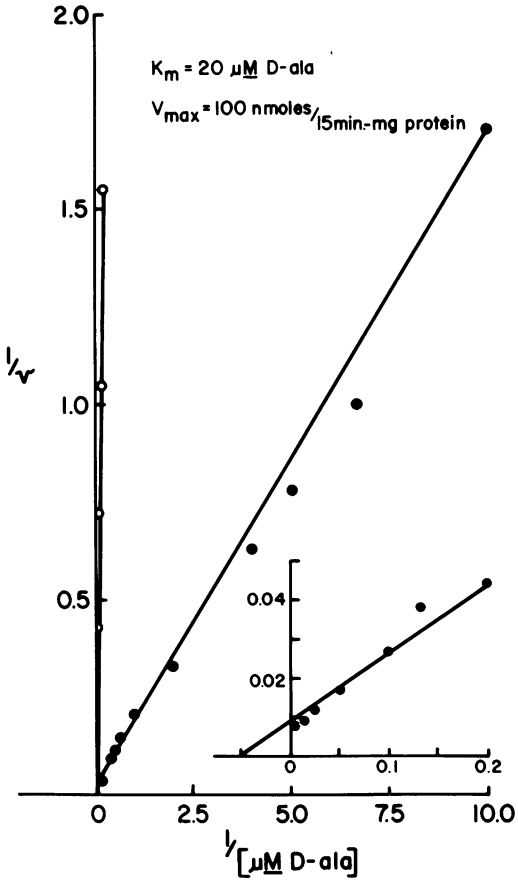

FIG. 2. *Lineweaver-Burk plot of net* D-*alanine transport. Velocity* (v) *is expressed as nmol per 15 min per mg of protein. Symbols:* ●, *the observed rate of uptake;* ○, *the rate of uptake that occurs in the presence of 10 mM sodium azide.*

show Lineweaver-Burk plots for the transport of these two amino acids, respectively. There appeared to be two K_m values for L-alanine, 1 μM and 10 μM, but only one K_m value for glycine transport, 20 μM. The kinetic parameters for the transport of these three amino acids are given in Table 1.

To determine whether D-alanine, L-alanine, and glycine were transported by the same systems, the ability of L-alanine and glycine to exchange for D-alanine was investigated (Fig. 5). Cells were allowed to accumulate 1 μM or 20 μM D-[^{14}C]alanine for 3.25 min, at which time excess D-alanine, L-alanine, glycine, D-cycloserine, or sodium azide was added. D-Alanine, L-alanine, and glycine caused exchange when added at a 1,000-fold excess. D-Cycloserine caused inhibition of further D-alanine accumulation when added at low concentrations (1.5 mM), but caused exchange when added at higher concentrations (75 mM). The simultaneous addition of a 1,000-fold excess of unlabeled D-alanine and 1.5 mM D-cycloserine produced an inhibition of exchange at 1 μM D-[^{14}C]alanine but not at 50 μM D-[^{14}C]alanine concentrations. The addition of sodium azide resulted in efflux at both D-[^{14}C]alanine concentrations.

To further explore the interdependence of the transport of D-alanine, L-alanine, and glycine, the inhibition of the transport of each of these amino acids by related compounds was determined. The inhibition of transport was determined at two concentrations of the amino acid, 1 μM and 20 μM. The inhibitor was added at two concentrations, 10 times the concentration of the amino acid transported and 1 mM. These two inhibitor concentrations were used to measure, respectively, the ability of the inhibitor to compete with the labeled amino acid when

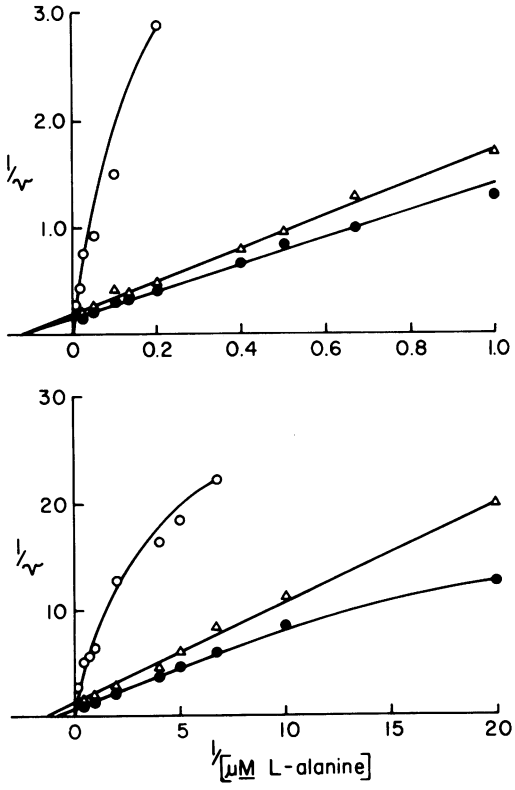

FIG. 3. *Lineweaver-Burk plots of* L-*alanine uptake. Velocity (v) is expressed as nmol/min per mg of protein. Symbols are the same as in the legend to Fig. 1.*

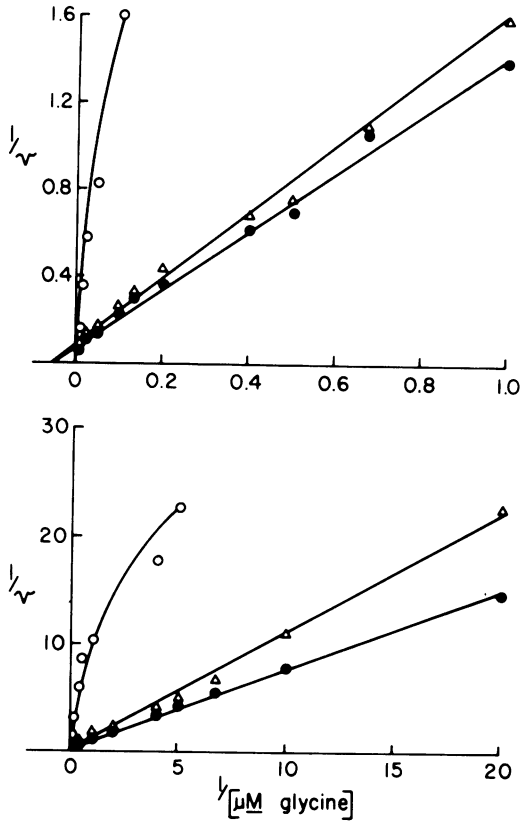

FIG. 4. *Lineweaver-Burk plots of glycine uptake. Velocity (v) is expressed as nmol/min per mg of protein. Symbols are the same as in the legend to Fig. 1.*

present in only slight excess and the maximal inhibition that is obtained.

D-Alanine transport (Table 2) was inhibited by L-alanine, glycine, and D-alanyl-D-alanine when the D-alanine concentration was 1 μM and the inhibitor concentration was 10 μM. Increasing the concentration of the inhibitors, D-cycloserine and D-serine, to 1 mM caused substantial inhibition. The same general pattern was seen when the D-alanine concentration was 20 μM, except that in this case D-serine inhibited substantially when present in 10-fold excess. L-Alanine was a more effective inhibitor of D-alanine transport than was D-alanine, and glycine was less effective than D-alanine. The dimer, D-alanyl-D-alanine, inhibited significantly when present in a 10-fold excess, but this inhibition was not greatly increased by an increase in D-alanyl-D-alanine concentration to 1 mM. The unrelated amino acid, proline, caused inhibition which appeared to be independent of proline concentration. Surprisingly, L-serine was a poorer inhibitor than proline.

TABLE 1. *Kinetic parameters of* D-*alanine,* L-*alanine, and glycine transport*

Amino acid	K_m (μM)	V_{max} (nmol/min per mg of protein)
D-Alanine	1	0.6
	20	10
L-Alanine	1	0.8
	10	5
Glycine	20	12

L-Alanine transport was substantially inhibited (Table 3) by L-alanine, D-alanine, glycine, D-cycloserine, and D-serine at 1 μM L-alanine and 1 mM inhibitor concentrations, but only unlabeled L-alanine was able to inhibit approximately 50% at a 10-fold excess. When the L-alanine concentration was increased to 20 μM, L-alanine, D-alanine, glycine, D-cycloserine, and D-serine all inhibited significantly at both inhibitor concentrations. The dimer, D-alanyl-D-alanine, caused a low amount of inhibition

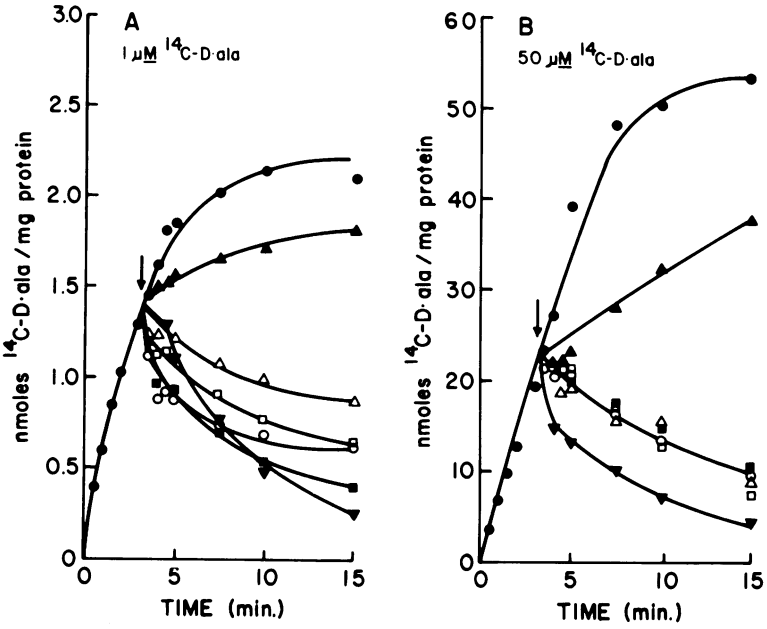

FIG. 5. *Efflux and exchange of* D-*alanine. Cells were allowed to accumulate* D-[^{14}C]*alanine at either of the two concentrations shown for 3.25 min, at which time the following additions were made: (A)* ●, *none;* ○, *plus 1 mM* D-[^{12}C]*alanine;* ▲, *plus 1.5 mM* D-*cycloserine;* △, *plus 1 mM* D-[^{12}C]*alanine plus 1.5 mM* D-*cycloserine;* ■, *plus 75 mM* D-*cycloserine;* □, *plus 1 mM* L-*alanine or 1 mM glycine;* ▼, *plus 10 mM sodium azide. (B)* ●, *none;* ○, *plus 50 mM* D-[^{12}C]*alanine;* ▲, *plus 1.5 mM* D-*cycloserine;* △, *plus 50 mM* D-[^{12}C]*alanine plus 1.5 mM* D-*cycloserine;* ■, *plus 75 mM* D-*cycloserine;* □, *plus 50 mM* L-*alanine or 50 mM glycine;* ▼, *plus 10 mM sodium azide.*

TABLE 2. *Inhibition of* D-*alanine transport*

Inhibitor	Concn	Inhibition (%) of D-alanine	
		1 μM	20 μM
None		(0.83)[a]	(8.4)[a]
D-Alanine	10×[b]	32	65
	1 mM	90	84
L-Alanine	10×	52	86
	1 mM	92	91
Glycine	10×	22	44
	1 mM	82	81
D-Cycloserine	10×	0	0
	1 mM	42	35
D-Serine	10×	5	57
	1 mM	86	80
D-Alanyl-D-alanine	10×	22	16
	1 mM	34	30
L-Serine	10×	7	0
	1 mM	35	0
Proline	10×	32	24
	1 mM	34	19
Azide	10 mM	82	86

[a] Velocity at this concentration is expressed as nmol/min per mg of protein.

[b] The concentration of inhibitor is 10 μM when the concentration of D-alanine is 1 μM and 200 μM when the concentration of D-alanine is 20 μM.

TABLE 3. *Inhibition of* L-*alanine transport*

Inhibitor	Concn	Inhibition (%) of L-alanine	
		1 μM	20 μM
None		(0.76)[a]	(4.8)[a]
L-Alanine	10×[b]	49	80
	1 mM	94	89
D-Alanine	10×	27	59
	1 mM	81	79
Glycine	10×	12	42
	1 mM	77	69
D-Cycloserine	10×	19	41
	1 mM	58	56
D-Serine	10×	3	43
	1 mM	76	68
D-Alanyl-D-alanine	10×	11	0
	1 mM	29	13
L-Serine	10×	21	0
	1 mM	41	29
Proline	10×	15	3
	1 mM	13	2
Azide	10 mM	90	84

[a] Velocity at this concentration is expressed as nmol/min per mg of protein.

[b] The concentration of inhibitor is 10 μM when the concentration of L-alanine is 1 μM and 200 μM when the concentration of L-alanine is 20 μM.

when the L-alanine concentration was 1 μM, but had little effect when the L-alanine concentration was 20 μM. The best inhibitor of L-alanine transport was L-alanine and D-alanine was the next most effective, followed by any of the amino acids in the group of glycine, D-cycloserine, and D-serine.

Glycine transport was inhibited (Table 4) by glycine, D-alanine, L-alanine, D-cycloserine, and D-serine to essentially identical extents at both glycine concentrations. There was very little inhibition of glycine transport by D-alanyl-D-alanine.

DISCUSSION

The transport of D-alanine and L-alanine appears to occur by two kinetically distinguishable systems. The high-affinity system, which has a K_m value of 1 μM D- or L-alanine and a V_{max} of 0.6 to 0.8 nmol/min per mg of protein, appears to be specific for these two amino acids. The structurally similar amino acids, glycine, D-cycloserine, and D-serine, are not able to compete effectively, as evidenced by their lower ability to inhibit when present in a 10-fold excess relative to the D- or L-alanine concentration. The dimer, D-alanyl-D-alanine, appeared to be able to inhibit D-alanine uptake when it was present in a 10-fold excess, and this inhibition was not substantially increased by increasing the D-alanyl-D-alanine concentration to 1 mM. The interpretation of this result is clouded by the observation that proline was more effective than the dimer at low concentrations of D-alanine. The dimer is not an effective inhibitor of alanine transport when the concentration of alanine is 20 μM. If it can be assumed that the two transport systems are completely independent of each other and that the rate of transport is linear with respect to substrate concentration at concentrations that are less than the K_m for transport, then calculations can be made based upon the kinetic parameters for transport given in Table 1 as to the percentage of amino acid transported by each system when the concentration of alanine is 1 μM.

TABLE 4. *Inhibition of glycine transport*

Inhibitor	Concn	Inhibition (%) of glycine	
		1 μM	20 μM
None		(0.73)[a]	(7.1)[a]
Glycine	10×[b]	28	57
	1 mM	86	83
D-Alanine	10×	35	77
	1 mM	85	85
L-Alanine	10×	30	60
	1 mM	86	89
D-Cycloserine	10×	23	69
	1 mM	83	82
D-Serine	10×	27	55
	1 mM	76	76
D-Alanyl-D-alanine	10×	3	1
	1 mM	19	0
L-Serine	10×	4	0
	1 mM	39	10
Proline	10×	19	0
	1 mM	20	0
Azide	10 mM	85	93

[a] Velocity at this concentration is expressed as nmol/min per mg of protein.

[b] The concentration of inhibitor is 10 μM when the concentration of glycine is 1 μM and 200 μM when the concentration of glycine is 20 μM.

Calculations for D-alanine transport were made as follows. In the high-affinity system, K_m equals 1 μM D-alanine and V_{max} equals 0.6 nmol/min per mg of protein. Therefore, when the D-alanine concentration is 1 μM, the velocity is 0.5 V_{max} or 0.3 nmol/min per mg of protein. In the low-affinity system, K_m equals 20 μM and V_{max} equals 10 nmol/min per mg of protein, so that when the D-alanine concentration is 20 μM, the velocity is 0.5 V_{max} or 5 nmol/min per mg of protein. If the rate is linear with respect to substrate concentration at D-alanine concentrations less than the K_m, then the velocity at 1 μM can be determined by dividing the rate at 20 μM by 20, yielding a velocity of 0.25 nmol/min per mg of protein. The total velocity at 1 μM is the sum of the velocities of the two systems, 0.55 nmol/min per mg of protein, and the contribution due to the high-affinity system is 0.30/0.55 × 100 = 55%. Similar calculations can be made for L-alanine transport, with a K_m equal to 1 μM, V_{max} equal to 0.8 nmol/min per mg of protein, and K_m equal to 10 μM, V_{max} equal to 5 nmol/min per mg of protein, as the kinetic parameters. In this case the high-affinity system contributes 0.4 nmol/min per mg of protein (60%) and the low-affinity system contributes 0.25 nmol/min per mg of protein (40%) for a total velocity of 0.65 nmol/min per mg of protein at 1 μM L-alanine.

At a 1 μM D-alanine concentration, 55% of the D-alanine is transported by the high-affinity system and 45% by the low-affinity system, and at a 1 μM L-alanine concentration, 60% of the L-alanine is transported by the high-affinity system and 40% by the low-affinity system. Therefore, a specific inhibitor of the high-affinity system would have a maximal inhibition capability of 55 and 60% for D- and L-alanine

uptake, respectively, and a specific inhibitor of the low-affinity system would have a maximal inhibition capability of 45 and 40% for D- and L-alanine uptake, respectively. L-Alanine appears to be the preferred amino acid for transport by the high-affinity system, as evidenced by the fact that L-alanine inhibits both D-alanine and L-alanine transport better than D-alanine or D-alanyl-D-alanine.

The low-affinity system (K_m of 20 μM for D-alanine and glycine and 10 μM for L-alanine, with a V_{max} of 5 to 12 nmol/min per mg of protein) appears to transport D-alanine, L-alanine, and glycine, based upon substrate saturation data, as well as D-cycloserine and D-serine, based upon transport inhibition data. Glycine, D-cycloserine, and D-serine are not effective inhibitors at 10-fold excess to the 1 μM concentration of transported amino acid, but are effective inhibitors when present at 1 mM or when the transported amino acids are at 20 μM. Presumably the inhibition observed when the inhibitor concentration is 1 mM could be due to exchange as well as to inhibition of uptake. The transport of D-alanine and L-alanine at 20 μM each is inhibited better by D-alanine and L-alanine than by glycine, D-cycloserine, and D-serine. This would be expected if part of the transport is due to the high-affinity system, which is not inhibited by the latter three amino acids. Glycine transport, on the other hand, is equally inhibited by glycine, D- and L-alanine, D-serine, and D-cycloserine, as would be expected if glycine was transported only by the low-affinity system. D-Serine is a less effective inhibitor, which may indicate that the transport system has a lower affinity for D-serine than for glycine. D-Alanyl-D-alanine is not effective as an inhibitor of glycine transport. Although this transport system shows little stereospecificity for D- and L-alanine, it is specific for the stereoisomers of serine. D-Serine is a good inhibitor of the low-affinity transport system, but L-serine is not. Transport of aromatic D-amino acids in *E. coli* (7) was found not to be stereospecific. Most systems (2, 5, 12), however, do show stereospecificity for serine.

Further evidence for the common transport systems for D- and L-alanine and glycine is the ability of L-alanine and glycine to cause D-alanine exchange. D-Cycloserine inhibits D-alanine uptake at both 1 and 50 μM D-alanine, and is capable of causing exchange only when present at a high concentration (75 mM). Therefore, D-cycloserine is probably transported by a low-affinity system that also transports D-alanine at a higher affinity. The D-alanine taken up into the cell is probably not chemically altered, since most of it can be effluxed from the cell by sodium azide.

Sodium azide, an uncoupler of electron transport, inhibits the transport of all three amino acids, D- and L-alanine and glycine. There appears to be a higher amount of azide insensitive uptake of D-alanine (Fig. 1) than of L-alanine (Fig. 3) or glycine (Fig. 4). In the Lineweaver-Burk plots, the reciprocal of the azide insensitive uptake extrapolates to zero, indicating that the K_m for this uptake extrapolates to infinity. This type of uptake is indicative of passive diffusion. We postulate that the deviation from linearity of the azide-insensitive transport at low substrate concentrations is due to binding of the substrate in the absence of transport. This suggestion is supported by the observation that D- and L-alanine can inhibit the uptake of D- and L-alanine better than azide can. Such a result would be expected if they are able to compete for binding as well as for transport.

In conclusion, there are two systems observed in whole cells of *B. subtilis* 168 for the transport of D-alanine, L-alanine, and glycine—a high-affinity system specific for the two stereoisomers of alanine, and a less specific, low-affinity system which transports D- and L-alanine, glycine, D-cycloserine, and D-serine. This transport can be inhibited by sodium azide, and azide can cause efflux. Since azide is an uncoupler of electron transport, its action indicates that the transport of these amino acids is probably linked to the electron transport chain as postulated by Kaback (4) and by Konings and Freese (6).

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