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Specificity of the Tyrosine-Phenylalanine Transport System in *Bacillus subtilis*

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L-Tyrosine and L-phenylalanine enter cells of *Bacillus subtilis* via a system of active transport that exhibits complex kinetic behavior. The specificity of the transport system was characterized both at low concentrations of transport substrate (where affinity for L-tyrosine or L-phenylalanine is high but capacity is low) and at high concentrations (where affinity is low but capacity is high). Specificity was not found to differ significantly as a function of either L-tyrosine or L-phenylalanine concentration. Kinetic analysis showed that the relationship between the uptake of L-phenylalanine and L-tyrosine is strictly competitive. Neither L-tyrosine nor L-phenylalanine uptake was competitively inhibited by other naturally occurring L-amino acids, indicating the importance of the phenyl side chain to uptake specificity. Hence, it is concluded that L-tyrosine and L-phenylalanine are transported by a common system that is specific for these two amino acids. The abilities of analogue derivatives of L-tyrosine and L-phenylalanine to inhibit the uptake of L-[^{14}C]tyrosine and L-[^{14}C]phenylalanine competitively were determined throughout a wide range of substrate and inhibitor concentrations. In this manner, the contributions of the side chain, the α-amino group and the carboxyl group to uptake specificity were established. It is concluded that the positively charged α-amino group contributes more significantly to uptake specificity than does the negatively charged carboxyl group. The recognition of a phenyl ring is an essential feature of specificity; other amino acids with aromatic side chains, such as the indole and imidazole rings of L-tryptophan and L-histidine, do not compete with L-tyrosine and L-phenylalanine for uptake. The presence of the *p*-hydroxy substituent in the side chain (as in L-tyrosine) enhances the uptake of the aryl amino acid analogues investigated.

The transport of small molecules into microbial cells has been studied extensively, most notably in the prokaryotic *Escherichia-Salmonella* and in the eukaryotic *Neurospora-Saccharomyces* organisms. The majority of studies involve kinetic and specificity analyses. Among the most impressive modern accomplishments in the field of microbial transport are the discovery of the phosphoenolpyruvate-dependent phosphotransferase system for hexose sugar transport by Roseman (21), the characterization of the M protein for β-galactoside transport by Fox et al. (9) and the development of the membrane vesicle system by Kaback (13).

The surprisingly sparse literature about transport in *Bacillus subtilis*, a major experimental system of biochemical genetics, includes studies of the uptake of citrate (24) and purines (5), a general survey of amino acid uptake (4), and characterization of an arginine permease (8). Information about a number of specific transport systems existing for amino acid transport was obtained recently with a membrane vesicle system in *B. subtilis* (16). Analysis of competitive inhibition of the transport of a given amino acid by other amino acids in the membrane vesicles of *B. subtilis* indicated that L-tyrosine and L-phenylalanine share a common transport system. L-Tyrosine and L-phenylalanine enter cells of *S. typhimurium* (1), *E. coli* (20), *N. crassa* (19), and *S. cerevisiae* (10) via multiple systems of transport, each system having distinctive specificity characteristics.

The design of affinity-labeling reagents that are highly selective for particular catalytic sites requires a knowledge of specificity characteristics. The tyrosine-phenylalanine system of transport in *B. subtilis* appears to possess characteristics of affinity and specificity that are suitable for the approach of affinity labeling (22). The present analysis provides data that

may aid in the design of affinity labels capable of covalent linkage to a specific binding site used for the transport of L-tyrosine and L-phenylalanine in *B. subtilis*. The success of this approach at the whole cell level would help resolve many questions about the physiological nature of the transport process.

MATERIALS AND METHODS

Organism. *B. subtilis* isolate H69, a prototrophic derivative of strain 168 (2), was used exclusively in these studies. Isolate H69 has a genetic background that prevents degradation of *N*-chloroacetyl derivatives of L-tyrosine or L-phenylalanine, probably owing to the loss of some proteolytic activity. Mutation to loss of ability to cleave *N*-acetyl groups from L-tyrosine or L-phenylalanine permits the accurate assessment of α-amino analogues of L-tyrosine and L-phenylalanine, as inhibitors of transport. Isolate H69 has the following history. The tyrosine auxotroph NP 35 (7), a nutritional mutant that can degrade *N*-chloroacetyl tyrosine to L-tyrosine, utilizing the L-tyrosine to satisfy the growth requirement, was mutagenized with 1-methyl-3-nitro-1-nitrosoguanidine as previously described (12). A survivor isolate which could not utilize *N*-chloroacetyl tyrosine to satisfy the L-tyrosine auxotrophy was cloned and purified. A spontaneous revertant to nutritional independence was selected from the auxotroph, cloned for purification, and assigned the isolate number H69.

Cultivation media. The composition of the minimal salts-glucose-trace elements medium was previously given (11). Solid medium contained the ingredients of liquid medium plus 1.5% (wt/vol) agar (Difco). Stock cultures were maintained on AK sporulation medium (Baltimore Biological Laboratories) at room temperature. Old cell populations were rejuvenated by growth on Trypticase soy agar (BBL) in overnight culture at 37 C prior to inoculation into minimal salts-glucose medium.

Media used in uptake experiments. Net increase in growth mass was prevented by nitrogen starvation after suspension of cells in minimal salts-glucose medium lacking ammonium sulfate.

Initial inoculum for transport experiments. A 200-ml culture grown in a 1-liter culture flask to an absorbance at 600 nm of about 0.5 (mid-exponential phase of growth) was harvested. The pellet obtained by centrifugation was resuspended in a 10-ml volume of fresh minimal salts-glucose medium. Portions of 1.0-ml were quick-frozen in liquid nitrogen and stored at −80 C in a Revco freezer until needed. These concentrated cell preparations did not require added glycerol to maintain viability. The replicate samples provided consistent inocula characteristics, yielding entirely comparable results over storage intervals of at least a month. Storage vials were thin-walled, plastic culture tubes (capped) from Scientific Products.

Uptake protocol. A storage vial containing frozen cells of strain H69 was thawed at room temperature and used to adjust 20 ml of minimal salts-glucose medium at 37 C to a turbidity of 13 Klett units (red filter) in a 125-ml side-arm flask. One milliliter of culture having an absorbance at 600 nm of 0.100 corresponds to a turbidity of 10 Klett units and 45 μg dry weight of cell mass. The cells were incubated at 37 C in a shaking water bath for about 4 h until the turbidity reached 45 Klett units. The cells were collected on a 47-mm membrane filter (Millipore Corp., 0.45-μm pore size) and suspended in 20 ml of medium lacking ammonium salts. The cell suspension was incubated in a 125-ml side-arm flask at 37 C with shaking for 30 min to deplete the amino acid pools. Then a 0.4-ml vol containing 4 mg of chloramphenicol was added to abolish protein synthesis occurring as a result of protein turnover. The cells were incubated an additional 30 min at 37 C with shaking.

Uptake was assayed by incubating 0.8-ml portions of nitrogen-starved, chloramphenicol-treated cells with 0.1 ml of L-[^{14}C]amino acid and 0.1 ml of solution of the unlabeled inhibitor, present at the final concentrations noted in the tables and figures. Under these conditions, uptake was linear for at least 5 min with L-[^{14}C]phenylalanine or L-[^{14}C]tyrosine. At the designated times, cell samples were collected on 25-mm Schleicher and Schuell membrane filters (0.45-μm pore size) and washed with 20 ml of the nitrogen-deficient medium (warmed to 37 C). The filters were placed in scintillation vials and dried under an infrared heat lamp. A 10-ml vol of Omnifluor scintillation fluid (New England Nuclear Corp.) was added, and the samples were counted for 5 min in a Beckman CPM liquid scintillation counter.

Chemicals. L-Proline, L-aspartic acid, and *p*-hydroxyphenylpyruvate were products of Mann Research Laboratories, Inc.; L-phenylalanine, *p*-aminophenylalanine, *p*-chlorophenylalanine, *p*-fluorophenylalanine, β-2-thienylalanine, *p*-hydroxyphenylacetate, chloramphenicol, *p*-hydroxyphenylpropionate, and tyramine were obtained from Sigma Chemical Corp.; all other L-α-amino acids were from Calbiochem. 3,4-Dihydroxyphenylalanine was obtained from Nutritional Biochemical Corp.; all other dihydroxy derivatives of phenylalanine and *p*-hydroxycinnamate were obtained from Aldrich Chemical Co. Toluene was from Baker Chemical Co. All other chemicals were of the best reagent grade commercially available. Uniformly labeled L-[^{14}C]phenylalanine in 50% ethanol (492 mCi/mmol) was purchased from Amersham/Searle. Uniformly labeled L-[^{14}C]-tyrosine in 0.1 N HCl (469 mCi/mmol) was obtained from New England Nuclear Co.

The *N*-acetyl and *N*-chloroacetyl derivatives of L-tyrosine and L-phenylalanine, products of Mann and Sigma Chemical Corp., respectively, were prepared free of chemical contaminants by passing an aqueous solution of the derivatives through a column of Sephadex A-50 (acid form) and lyophilizing the eluate. *N*-chloroacetyl leucine, obtained from Mann, and *N*-chloroacetyl tyramine, obtained from Sigma Chemical Corp., were purified in the manner described above.

Preparation of 3-amino-1-chloro-4-(4-hydroxyphenyl)-2-butanone hydrochloride (tyrosine chloromethyl ketone). A 4.2-g amount (20 mmol) of phosphorous pentachloride was added, with stirring,

to 8.7 g (19.4 mmol) of *N*, *O*-dicarbobenzoxy tyrosine (14) in 40 ml of absolute tetrahydrofuran at 0 C. After 20 min at 0 C, the solution was gradually added (within several minutes) to an ice-cooled solution of excess (about 6 g) diazomethane (prepared from Diazald according to literature from the Aldrich Chemical Co.) in 400 ml of ether. After about 15 min of additional stirring, the evolution of nitrogen was complete, and dry hydrogen chloride gas was slowly bubbled into the solution for 15 min. After concentration of the mixture in vacuo, the residual oil was dissolved in a minimum of acetone, and the product was precipitated by addition of petroleum ether (bp 30–60 C). The yellow solid was recrystallized from methanol, yielding pale yellow crystals of *N*, *O*-dicarbobenzoxy tyrosine chloromethyl ketone (mp 122–124 C; nuclear magnetic resonance (NMR) spectrum (acetone-D_6, δ values): 2.8–3.2 (multiplet, 2 H), 4.4–4.7 (multiplet, 1 H), 4.5 (singlet, 2 H), 5.0 (singlet, 2 H), 5.2 (singlet, 2 H), 7.0–7.6 (complex multiplet, 15 H).

Analysis calculated for $C_{24}H_{24}NO_6Cl$ was: C, 63.00; H, 5.28; N, 3.06. The values determined were: C, 63.87; H, 5.5; N, 2.84.

N, *O*-dicarbobenzoxy tyrosine chloromethyl ketone (4.4 g [9.1 mmol]) was deblocked by refluxing in 30 ml of dry trifluoroacetic acid under a stream of dry hydrogen chloride gas for 15 min. The resultant dark-brown solution was evaporated to an orange oil by placing it in an evacuated desiccator over sodium hydroxide pellets. The residue was taken up in 200 ml of water and extracted with three 50-ml portions of methylene chloride. Residual methylene chloride was removed from the aqueous phase by rotary evaporation in vacuo. The solution was passed through a column (34 by 2.5 cm) of SE-Sephadex, C-25, in the acid form, and the chloromethyl ketone was retained by the column. The column was washed with 600 ml of water, and the chloromethyl ketone hydrochloride was eluted with 0.05 M hydrochloric acid (the chloromethyl ketone was eluted between 600–700 ml). The aqueous solutions were concentrated in vacuo to a light-yellow solid, 1.2 g. The product could not be recrystallized without decomposition. The elemental analysis was not satisfactory although it was close to theoretical values. However, the NMR spectrum was clearly consistent with the expected chloromethyl ketone: NMR (CF_3COOH, δ values): 3.4 (singlet, 2 H), 4.4 (singlet, 2 H), 5.0–5.2 (broad singlet, 1 H), 7.0–7.65 (complex multiplet, 8 H).

The chloromethyl ketone derived from phenylalanine (23) and the chloromethyl ketone derived from leucine (6) were prepared as described in the references cited. The bromomethyl ketone derived from phenylalanine was the generous gift of Elliott N. Shaw.

RESULTS

Kinetic features of the uptake system. Measurements of the uptake of L-tyrosine or L-phenylalanine into whole cells of *B. subtilis* were done at exogenous concentrations ranging from 1 to 100 μM. Uptake velocities of both L-tyrosine and L-phenylalanine exhibited reasonably good proportionality with respect to uptake time. The curves given in Fig. 1 illustrate uptake as a function of time at low (4 μM) and high (100 μM) concentrations of L-tyrosine or L-phenylalanine. More exact determinations of initial velocities were not necessary to the objectives of this study. The variation of transport velocity as a function of L-tyrosine or L-phenylalanine concentrations extends over a very large range. Graphical presentation of the data in double reciprocal form reveals the biphasic dependence of transport velocity upon either L-tyrosine (Fig. 2) or L-phenylalanine concentration (Fig. 3). Inhibition of L-[^{14}C]-tyrosine transport by L-phenylalanine was strictly competitive as shown graphically (Fig. 2) in either region of the biphasic saturation curve. When L-[^{14}C]phenylalanine transport

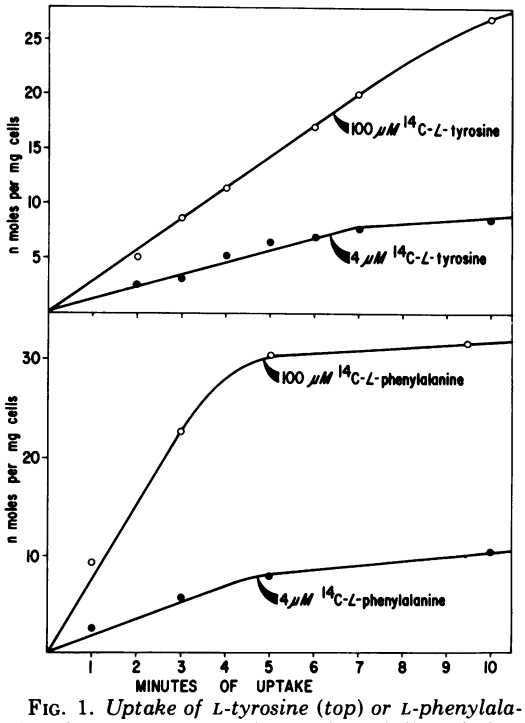

FIG. 1. *Uptake of* L-*tyrosine (top) or* L-*phenylalanine (bottom) as a function of time. Cells of isolate H69 (nitrogen starved and chloramphenicol treated as described in Materials and Methods) were assayed for uptake of* L-[^{14}C]*tyrosine or* L-[^{14}C]*phenylalanine at 37 C as described in Materials and Methods. Low (4 μM) and high (100 μM) concentrations of* L-[^{14}C]-*tyrosine or* L-[^{14}C]*phenylalanine, corresponding to the two kinetically distinguishable modes of transport shown in Fig. 2 and 3, were used. Uptake velocities are expressed on the ordinate scales as nanomoles of* L-[^{14}C]*tyrosine or* L-[^{14}C]*phenylalanine taken up per milligram of cell dry weight.*

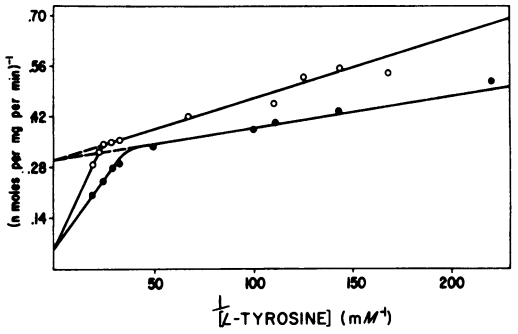

FIG. 2. *Double-reciprocal plot of* L-*tyrosine uptake into cells of isolate H69. Uptake velocity is expressed on the ordinate scale as the reciprocal of velocity (nanomoles of* L-[^{14}C]*tyrosine taken up per milligram of cell dry weight per minute) at 37 C and is plotted as a function of the reciprocal of* L-[^{14}C]*tyrosine concentration in the absence* (●) *or presence* (○) *of 0.1 mM* L-*phenylalanine. Other procedural details are given in Materials and Methods. Apparent values of* V_{max} *for* L-*tyrosine entry were 3.3 and 17.9 nmol per mg (dry wt) per min for the high and low affinity systems, respectively. The corresponding* K_m *values were 3.4 and 143 μM, respectively; the corresponding* K_i values were 135 and 251 *μM, respectively.*

was inhibited by L-tyrosine, the inhibition was again strictly competitive (Fig. 3).

Kinetic evaluation of the data is consistent with interpretations involving either: (i) a mixture of two independent transport systems, each recognizing both L-tyrosine and L-phenylalanine, or (ii) a single system exhibiting negative cooperative interactions (17) exerted between substrate-binding components of the transport system. Regardless of which interpretation is correct, specificity characteristics might differ depending upon whether assays are done at low concentrations of transport substrate (i.e., measuring the high affinity-low capacity system) or at high concentrations (i.e., measuring the low affinity-high capacity system). Hence, most experimental protocols called for inhibitor analysis at three concentrations of L-[^{14}C]tyrosine or L-[^{14}C]phenylalanine (i.e., 1, 10, and 100 μM). The ratios of test inhibitor to ^{14}C-labeled substrate in a given test series were ordinarily 1, 10, and 100. Since the results to follow indicate that L-tyrosine and L-phenylalanine share a common system(s) for their transport, the effects of various inhibitors on L-phenylalanine and L-tyrosine uptake are presented together (Tables 1-6) for a more convenient comparison.

Effect of 4-substituted phenylalanines. The uptake of both L-phenylalanine and L-tyrosine was substantially decreased by analogues having halo substituents at the 4-position of the L-phenylalanine ring. Substitution of an amino group at the 4-position led to much less inhibition of L-phenylalanine or L-tyrosine uptake (Table 1). β-2-thienylalanine (also included in Table 1) mimicked L-phenylalanine closely as a transport substrate. Appropriate kinetic analyses verified that all of the analogues tested in Table 1 were competitive inhibitors of L-phenylalanine and L-tyrosine uptake.

Effects of amino acids. The common L-α-amino acids were also tested as inhibitors. A detailed set of data are given in Table 2 to indicate the inhibitory capabilities of L-leucine (neutral), L-aspartate (acidic), and L-arginine (basic). Of these, L-leucine is an excellent inhibitor of both L-tyrosine and L-phenylalanine uptake. L-Aspartate is a fairly good inhibitor, while L-arginine is a poor inhibitor. At the highest concentrations of L-[^{14}C]phenylalanine or L-[^{14}C] tyrosine used, a characteristic and significant increase in inhibition of uptake by other amino acids was observed. These inhibitions are noncompetitive, and the effect is the greatest for the low affinity-high capacity system having the larger V_{max}.

The ability of other L-amino acids to inhibit the uptake of L-tyrosine (along with some data for L-phenylalanine) is summarized in Table 3. Inhibition by L-glutamate resembled that of

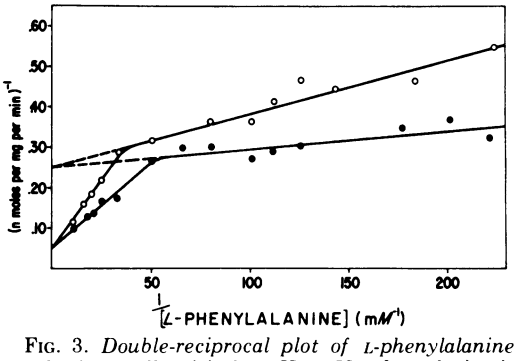

FIG. 3. *Double-reciprocal plot of* L-*phenylalanine uptake into cells of isolate H69. Uptake velocity is expressed on the ordinate scale as the reciprocal of velocity (nanomoles of* L-[^{14}C]*phenylalanine taken up per milligram of cell dry weight per minute) at 37 C and is plotted as a function of the reciprocal of* L-[^{14}C]*phenylalanine concentration in the absence* (●) *or presence* (○) *of 0.1 mM* L-*tyrosine. Other procedural details are given in Materials and Methods. Apparent values of* V_{max} *for* L-*phenylalanine entry were 4.0 and 20.0 nmol per mg (dry weight) per min for the high and low affinity systems, respectively. The corresponding* K_m *values were 2.1 and 80 μM, respectively; the corresponding* K_i *values were 60.2 and 89.9 μM, respectively.*

L-aspartate (Table 2). Among the remaining amino acids, L-threonine, L-isoleucine, L-valine, and L-cysteine were among the most effective inhibitors (all noncompetitive). L-Alanine was distinctly more inhibitory to L-phenylalanine uptake than to L-tyrosine uptake. The effect of amino acids (other than alanine) on L-phenylalanine uptake was similar to the effects noted on L-tyrosine uptake. L-Tryptophan and L-histidine were among the poorest inhibitors of L-tyrosine or L-phenylalanine uptake.

Contribution of α-amino or carboxyl group to binding. The *N*-acetyl and *N*-chloroacetyl derivatives of L-tyrosine are qualitatively better as inhibitors of L-[^{14}C]tyrosine uptake than of L-[^{14}C]phenylalanine uptake (Table 4). On the other hand, the two corresponding *N*-acyl derivatives of phenylalanine are poor inhibitors of both L-phenylalanine and L-tyrosine uptake. The *N*-acyl derivatives of other amino acids were equal to or less effective than the parent compounds as inhibitors (D'Ambrosio, unpublished results).

When the carboxyl group of L-tyrosine or L-phenylalanine was replaced by an uncharged ketone, e.g., tyrosine chloromethyl ketone, phenylalanine chloromethyl ketone, or phenylalanine bromomethyl ketone (Table 4), inhibition of L-[^{14}C]tyrosine uptake resembled that exhibited by L-phenylalanine (Table 1). On the other hand, these carboxy-modified derivatives were relatively poor inhibitors of L-[^{14}C]-phenylalanine uptake. The carboxyl-modified derivative of leucine, leucine chloromethyl ketone, resembled L-leucine (Table 2) in ability to inhibit (noncompetitively) L-tyrosine or L-phenylalanine uptake.

Considerable loss of binding accompanied the absence of the α-amino group in analogues of L-tyrosine, e.g., *p*-hydroxyphenylacetate, *p*-hydroxycinnamate, and *p*-hydroxyphenylpropionate (Table 5). Tyramine, lacking a carboxyl group, is an ineffective inhibitor, as is its *N*-chloroacetyl derivative. The addition of a second hydroxyl substituent at the meta position of the aromatic ring of L-tyrosine, i.e., 3,4-dihydroxyphenylalanine, decreases inhibition of L-[^{14}C]tyrosine or L-[^{14}C]phenylalanine uptake (Table 6), yielding results resembling those obtained with *p*-aminophenylalanine

TABLE 1. *Inhibition of L-tyrosine and L-phenylalanine transport by analogue derivatives*

Transport substrate[a]	Inhibitor-substrate ratio[b]	Inhibition (%) produced by:					
		L-Phenylalanine	L-Tyrosine	*p*-Aminophenylalanine	*p*-Fluorophenylalanine	*p*-Chlorophenylalanine	β-2-Thienylalanine
1 μM L-[^{14}C]Tyrosine	1	15	36	7	27	28	7
	10	43	88	26	50	43	33
	100	54	97	46	57	62	53
10 μM L-[^{14}C]Tyrosine	1	22	48	10	27	21	31
	10	50	85	29	44	50	46
	100	73	96	58	77	77	60
100 μM L-[^{14}C]Tyrosine	1	48	27	22	27	49	32
	10	70	63	38	55	65	53
	100			63	69	77	64
1 μM L-[^{14}C]Phenylalanine	1	49	17	10	30	17	25
	10	82	32	14	69	36	65
	100	92	51	27	86	59	87
10 μM L-[^{14}C]Phenylalanine	1	43	30	21	32	27	31
	10	75	41	23	71	50	79
	100	90	51	56	81	76	95
100 μM L-[^{14}C]Phenylalanine	1	32	20	12	27	28	54
	10	72	29	19	51	47	82
	100			48	86	80	93

[a] The uptake of L-[^{14}C]tyrosine or L-[^{14}C]phenylalanine was measured at three concentrations extending over a 100-fold range of the biphasic saturation curves (Fig. 2 and 3).

[b] Unlabeled inhibitor molecules were present at 1, 10, or 100 times the concentration of radiolabeled transport substrate.

TABLE 2. *Effect of leucine, aspartate, and arginine upon L-tyrosine and L-phenylalanine transport*

Transport substrate[a]	Inhibitor-substrate ratio[b]	Inhibition (%) produced by:		
		L-Leucine	L-Aspartate	L-Arginine
1 μM L-[^{14}C]Tyrosine	1	13	7	0
	10	32	12	5
	100	53	27	8
10 μM L-[^{14}C]Tyrosine	1	14	10	0
	10	38	24	0
	100	59	53	0
100 μM L-[^{14}C]Tyrosine	1	44	33	9
	10	56	68	21
	100	77	74	30
1 μM L-[^{14}C]Phenylalanine	1	20	10	1
	10	32	15	4
	100	45	18	5
10 μM L-[^{14}C]Phenylalanine	1	38	23	0
	10	45	28	1
	100	56	34	3
100 μM L-[^{14}C]Phenylalanine	1	64	33	14
	10	77	61	30
	100	87	68	33

[a] The uptake of L-[^{14}C]tyrosine or L-[^{14}C]phenylalanine was measured a three concentrations extending over a 100-fold range of the biphasic saturation curves (Fig. 2-3).

[b] Unlabeled inhibitor molecules were present at 1, 10, or 100 times the concentration of radiolabeled transport substrate.

(Table 1). The transport system exhibited little specificity for all of the dihydroxy analogues of L-phenylalanine lacking either an α-amino or a carboxyl group (Table 6).

DISCUSSION

Permease multiplicity appears to be a common mechanism providing microorganisms with the capacity to deal efficiently with very broad concentration ranges of one or more exogenous amino acids (1, 8, 13, 15, 18). The physiological utility of such mixed systems seems apparent. One component is frequently a high affinity-low capacity uptake system that allows cells to scavenge and concentrate low concentrations of extracellular amino acids for utilization in protein synthesis. A second component may be a low affinity-high capacity uptake system, appropriate to the utilization of the greater amounts of amino acids required for use as a carbon and energy and/or nitrogen source. Usually the high capacity system of transport is less specific than the high affinity system.

The existence of a mixed system of transport in *B. subtilis* for L-tyrosine and L-phenylalanine uptake is probably the most obvious interpretation of the biphasic dependence of transport velocity upon L-tyrosine or L-phenylalanine concentration. In the case of a mixed system, correction of the kinetic parameters for transport by the high affinity system at high substrate concentration would yield K_m values that differ by a factor of about 2 with little effect on the stated V_{max} values. However, an alternative interpretation of the biphasic kinetics could involve a single system exhibiting negative cooperativity (17). The failure of a variety of nutritional conditions to alter the ratio of the two kinetically defined transport phases (S. Stenmark and R. Jensen, unpublished observations) as well as the lack of specificity difference between them furnish tentative support to a negative cooperative model. Further information is necessary to decide between these mechanisms. One approach to the differentiation of these possibilities involves data about the heterogeneity of the protein. The difficulty in applying this criterion to membrane-bound transport systems is that one protein of a heterogeneous mixture could be lost, e.g., owing to denaturation or simply to lack of

TABLE 3. *Effect of L-amino acids upon L-tyrosine and L-phenylalanine uptake*

Inhibitor	Inhibition (%)					
	10 μM L-[^{14}C]Tyrosine[a]			10 μM L-[^{14}C]Phenylalanine[a]		
	1[b]	10[b]	100[b]	1[b]	10[b]	100[b]
L-Alanine	0	14	25	27	36	47
L-Cysteine	15	36	61			
L-Glutamate	18	42	52			
Glycine	18	21	37			
L-Histidine	4	6	11	9	19	25
L-Isoleucine	23	29	38			
L-Lysine	4	4	5	9	14	17
L-Methionine	2	7	25			
L-Proline	10	22	36			
L-Hydroxyproline	0	14	25			
L-Serine	12	32	46			
L-Threonine	32	38	43	30	40	47
L-Tryptophan	11	17	21	0	12	21
L-Valine	19	30	39			

[a] Transport substrate.

[b] Inhibitor-substrate ratio. Unlabeled amino acids under test as inhibitors were present at 1, 10, or 100 times the concentration of L-[^{14}C]tyrosine (left) or L-[^{14}C]phenylalanine (right). The decreased uptake observed in the presence of inhibitor is expressed as percent inhibition.

TABLE 4. *Effect of α-amino or L-carboxyl modification on transport of L-tyrosine and L-phenylalanine*

Transport substrate[a]	Inhibitor-substrate ratio[b]	Inhibition (%) produced by:							
		N-Acetyl phenylalanine	*N*-Chloroacetyl phenylalanine	*N*-Acetyl tyrosine	*N*-Chloroacetyl tyrosine	Tyrosine chloromethyl ketone	Phenylalanine chloromethyl ketone	Phenylalanine bromomethyl ketone	Leucine chloromethyl ketone
1 μM L-[^{14}C]Tyrosine	1	0	11	18	9	13	7		7
	10	8	20	26	45	29	14		17
	100	14	39	45	54	51	38		38
10 μM L-[^{14}C]Tyrosine	1	3	0	17	35	17	17	32	17
	10	6	12	48	56	35	48	45	34
	100	13	22	76	70	71	58	69	48
100 μM L-[^{14}C]Tyrosine	1	0	1	42	42	30	22		37
	10	5	17	52	51	44	37		45
	100	9	35	71		75			73
1 μM L-[^{14}C]Phenylalanine	1	9	10	10	4	0	4		0
	10	13	24	20	15	19	10		2
	100	55	40	31	31	26	26		26
10 μM L-[^{14}C]Phenylalanine	1	16	11	24	17	6	0	11	17
	10	19	13	32	30	10	20	34	41
	100	21	21	45	35	24	34	53	53
100 μM L-[^{14}C]Phenylalanine	1	0	6	6	0	19	25		66
	10	5	12	21	17	30	31		71
	100	5		37	33	58			81

[a] The uptake of L-[^{14}C]tyrosine or L-[^{14}C]phenylalanine was measured at three concentrations extending over a 100-fold range of the biphasic saturation curves (Fig. 2–3).

[b] Unlabeled inhibitor molecules were present at 1, 10, or 100 times the concentration of radiolabeled transport substrate.

binding of substrate by the membrane-free protein. Application of affinity labeling, leading to a covalently bound radioactive label, would allow positive identification of any transport protein initially involved in binding. The design of appropriate affinity-labeling reagents requires a knowledge of the structural features of the substrates that contribute to binding. Fortunately, derivatives of phenylalanine and tyrosine are available that are not only suitable for specificity studies but are also potential affinity-labeling reagents. Significant variations in specificity are not observed when different concentrations of L-tyrosine or L-phenylalanine are used in uptake experiments. L-Tyrosine and L-phenylalanine seem to compete with one another for the same binding sites, independent of concentration; inhibition of the transport of each by the other is strictly competitive. Hence, the complexity indicated by the biphasic kinetics of uptake does not appear to reflect differing specificities. The existence of a common system(s) for the transport of L-tyrosine and L-phenylalanine in *B. subtilis* is consistent with data obtained using a membrane vesicle system (16). It is of interest that the kinetic characteristics of the whole cell and membrane vesicle systems are similar for L-tyrosine and L-phenylalanine transport. No kinetic data have been reported for the membrane vesicle system at relatively high concentrations of transport substrate (16). A distinct tendency for greater inhibition of uptake by L-amino acids, e.g., aspartate or leucine, is noted when high concentrations of L-tyrosine or L-phenylalanine are used. However, these inhibitions are noncompetitive (D'Ambrosio, unpublished results) and therefore do not reflect characteristics of the site(s) under study. The noncompetitive inhibitions may reflect such phenomena as allosteric effects, displacement of the L-tyrosine-L-phenylalanine pool capacity from the "inside," or accentuated efflux.

The lack of specificity of the L-tyrosine-L-phenylalanine system for L-tryptophan and L-histidine contrasts sharply with the general

TABLE 5. *Effect of miscellaneous analogues upon L-tyrosine and L-phenylalanine uptake*

Inhibitor	Inhibition (%)					
	10 μM L-[^{14}C]Tyrosine[a]			10 μM L-[^{14}C]Phenylalanine[a]		
	1[b]	10[b]	100[b]	1[b]	10[b]	100[b]
p-Hydroxyphenylpyruvate	17	25	58			
p-Hydroxycinnamate	20	28	32			
p-Hydroxyphenylacetate	0	6	9	0	6	20
p-Hydroxyphenylpropionate	3	8	11			
α-Aminophenylacetate	13	24	44	27	35	37
Tyramine	0	0	8	2	6	24
N-Chloroacetyltyramine	0	0	0			
N-Chloroacetylleucine	18	27	39			

[a] Transport substrate.

[b] Inhibitor-substrate ratio. Unlabeled compounds under test as inhibitors were present at 1, 10, or 100 times the concentration of L-[^{14}C]tyrosine (left) or L-[^{14}C]phenylalanine (right). The decreased uptake observed in the presence of inhibitor is expressed as percent inhibition.

aromatic permease of *S. typhimurium* (1). The bulky side chain of L-tryptophan probably accounts for poor binding owing to steric factors. The hydrophilic nature of the imidazole ring of L-histidine and the structural difference introduced by replacement of the *p*-hydroxyl group of L-tyrosine by the *p*-amino group of *p*-aminophenylalanine probably account for reduced binding to the site that is specific for neutral hydrophobic aromatic rings. Possible effects depending upon positively charged side chains of L-histidine and *p*-aminophenylalanine seem unlikely at the neutral pH values used. The hydrophobic nature of the binding site is further indicated by the good inhibition of L-tyrosine and L-phenylalanine uptake by β-2-thienylalanine, a compound having a hydrophobic aromatic side chain.

Our results indicate that the positively charged α-amino group is essential to uptake specificity; on the other hand, the negatively charged carboxyl group can be converted to a neutral carbonyl group with only moderate losses in uptake. *N*-Acetyl and *N*-chloroacetyl phenylalanine are poor inhibitors of aromatic amino acid uptake. Steric inhibition of binding owing to the increased bulk at the α-amino group cannot account for the reduced uptake, since *N*-acetyl and *N*-chloroacetyl tyrosine are good competitive inhibitors. Furthermore, structural analogues of aromatic α-amino acids lacking the α-amino group altogether, such as *p*-hydroxyphenylacetic and *p*-hydroxycinnamic acids, are poor inhibitors of uptake. The *p*-hydroxyl group of the *N*-acyl tyrosine derivatives could compensate for the loss of the positive charge on the α-amino group by serving as an acceptor for a neutral hydrogen bond donor in the binding site for the side chain. However, it is also evident that the *p*-hydroxy group of other tyrosine analogues (Table 5) cannot compensate for decreased uptake when either the carboxyl group or α-amino group is not present, suggesting that even the uncharged amino and carboxyl derivatives may contribute to binding (probably through hydrogen bonding interactions).

Modification of the negatively charged car-

TABLE 6. *Effect of dihydroxy analogues of phenylalanine upon L-tyrosine and L-phenylalanine uptake*

Inhibitor	Inhibition (%)					
	10 μM L-[^{14}C]Tyrosine[a]			10 μM L-[^{14}C]Phenylalanine[a]		
	1[b]	10[b]	100[b]	1[b]	10[b]	100[b]
3,4-Dihydroxyphenylalanine	6	30	70	18	26	40
2,5-Dihydroxybenzaldehyde	5	10	12			
2,5-Dihydroxybenzoate	4	5	8			
3,5-Dihydroxybenzoate	0	3	10			
2,5-Dihydroxyphenylacetate	0	4	8			
2,3-Dihydroxybenzoate	11	14	24			
α-Chloro-3′,4′-dihydroxyacetophenon	22	24	40			
3-Hydroxytyramine	8	15	18			
3,4-Dihydroxybenzoate	0	9	11			
3,4-Dihydroxyphenylacetate	0	11	35	17	27	31
3,4-Dihydroxycinnamate	0	1	6	11	15	23

[a] Transport substrate.

[b] Inhibitor-substrate ratio. Unlabeled compounds under test as inhibitors were present at 1, 10, or 100 times the concentration of L-[^{14}C]tyrosine (left) or L-[^{14}C]phenylalanine (right). The decreased uptake observed in the presence of inhibitor is expressed as percent inhibition.

boxyl groups of L-tyrosine and L-phenylalanine to give neutral halomethyl ketones produced the greatest differentiation between L-tyrosine and L-phenylalanine uptake. Uptake of L-tyrosine was inhibited significantly more than L-phenylalanine by the chloromethyl ketone derivatives of tyrosine and pheynlalanine or by phenylalanine bromomethyl ketone. Absence of a carbonyl group, as in tyramine or *N*-chloroacetyl tyramine, decreased uptake markedly; the uncharged carbonyl group could be serving as an acceptor for a hydrogen bond donor located in the carboxylate binding site. This contribution to binding may partially compensate for the loss of negative charge. Carboxyl modifications resulting in the loss of charge do not lead to the drastic effects on uptake that attend amino modifications leading to loss of charge.

B. subtilis possesses a transport system that is highly specific for the side chains of L-tyrosine and L-phenylalanine. It is also clear that the positively charged amino group is important for uptake specificity. Modification of the carboxyl group of either of these amino acids to form the chloromethyl or bromomethyl ketone derivatives does not seriously reduce uptake. Therefore, these halomethyl ketones are excellent candidates as affinity labeling reagents that may permit the specific marking and subsequent physicochemical characterization of the aromatic amino acid binding protein(s) of *B. subtilis*.

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