pH-dependent Heterogeneity of Acidic Amino Acid Transport in Rabbit Jejunal Brush Border Membrane Vesicles*

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Initial rates of Na+-dependent L-glutamic and D-aspartic acid uptake were determined at various substrate concentrations using a fast sampling, rapid filtration apparatus, and the resulting data were analyzed by nonlinear computer fitting to various transport models. At pH 6.0, L-glutamic acid transport was best accounted for by the presence of both high (Km = 61 μM) and low (Km = 7.0 mM) affinity pathways, whereas D-aspartic acid transport was restricted to a single high affinity route (Km = 80 μM). Excess D-aspartic and L-phenylalanine served to isolate L-glutamic acid flux through the remaining low and high affinity systems, respectively. Inhibition studies of other amino acids and analogs allowed us to identify the high affinity pathway as the Xc(-) and the low affinity one as the intestinal NBB system. The pH dependences of the high and low affinity pathways of L-glutamic acid transport also allowed us to establish some relationship between the NBB and the more classical ASC system. Finally, these studies also revealed a heterotropic activation of the intestinal Xc(-) transport system by all neutral amino acids but glycine through an apparent activation of Vmax.

The early studies of Gibson and Wiseman (1) have provided the first evidence that, in the rat small intestine, the uptake of acidic amino acids is a carrier-mediated process. Since this work, a substantial body of evidence, as recently reviewed (2, 3), has accumulated showing a multiplicity of Na+-dependent and Na+-independent transport pathways for this class of amino acids in mammalian cells and tissues. Heterogeneity in Na+-dependent acidic amino acid transport systems has also been described in numerous cell types (2-4). For example, both high and low affinity systems for L-glutamic acid transport have been reported in membrane preparations of the central nervous system (2-4), in cultured human skin fibroblasts (5) and rat hepatocytes (6), and in rat (7) and rabbit (8) renal brush border membrane vesicles.

In the brush border membrane of the small intestine, contradictory results have appeared with regard to the heterogeneity of Na+-dependent acidic amino acid transport (2). For example, Lerner and Steinke (9) have reported a single transport system with a Km of 4-8 μM for the Na+-dependent uptake of L-glutamic acid across isolated segments of chick intestine. In this study however, whereas the uptake of 50 μM L-glutamic acid was markedly inhibited by D-aspartic acid and L-γ-methylglutamic acid, it was also partially inhibited by a number of neutral amino acids such as L-proline, L-leucine, and L-alanine. More recent studies by Wingrove and Kimmich (10) did show the presence of both high (Km = 16 μM) and low affinity (Km = 2.7 mM) routes for the Na+-dependent transport of L-aspartic acid in a preparation of isolated chick intestinal epithelial cells. Similarly, in studies using intestinal brush border membrane vesicles, Corelli et al. (11) found a single transport system in the rat with a Km of 1.5 mM for L-glutamic acid and 1.0 mM for L-aspartic acid whereas, in human, Rajendran et al. (12) demonstrated the presence of a high affinity transport for L-glutamic acid with a Km of 91 μM with no evidence for a low affinity transport pathway.

Transport studies that have examined the pH dependence of acidic amino acid transport have also led to contradictory results when performed under Na+ gradient conditions alone but seem to agree quite closely when done under optimum gradient conditions of both inward Na+ and outward K+ (2). This observation, in conjunction with the fact that a transport system for neutral amino acids with properties similar to those described for system ASC, according to Christensen's nomenclature (13), may serve upon protonation as a low affinity pathway for acidic amino acids in those cells types for which inhibitor specificity and pH dependence have been studied (14-16), could actually provide a rationale for the lack of consistency in the characterization of intestinal acidic amino acid transport pathways. Accordingly, the presence in the brush border membrane of the small intestine of both an ASC-like transporter for neutral amino acids and a more specific, Xc(-) type (13) of acidic amino acid transporter could have led to different results in the characterization of the acidic amino acid transport routes depending on the pH conditions of the uptake assay.

In this study, we have tested this hypothesis by determining the kinetics of both L-glutamic and D-aspartic acids under slightly acidic conditions (pH 6.0). The choice for these substrates was dictated quite naturally by the demonstration that the high affinity acidic amino acid transport system Xc(-) displays no stereospecificity between L- and D-aspartic acids, whereas ASC-like systems never accept D-aspartic acid as substrate (13). Since transport heterogeneity was observed in the case of L-glutamic acid only, we then tried to delineate the two transport pathways by inhibition studies. Finally, we report the pH dependence of the two transport pathways. All together, these results do support the presence of both ASC- and Xc(-)-like systems in the rabbit jejunal brush border membrane.

MATERIALS AND METHODS

Preparation of Brush Border Membrane Vesicles—Two large batches of rabbit jejunal brush border membrane vesicles were pre-
pared, as described recently (17), using a modified homogenate media in combination with Mg$^{2+}$ precipitation to ensure both vesicle pre-equilibration and stabilization. Briefly, for each batch of vesicles, the jejunum of pH 7.4 was isolated and flushed with ice-cold saline. The mucosal scrapings were homogenized in a 20:1 ratio of vesicle preparation, a suitable number of aliquots were thawed and resuspended in the media required for the particular experiment (see descriptions in the figure and table legends) and prepared down to the final vesicle pellet (P1). The vesicles, resuspended in the media to give a final concentration of about 25 mg of protein/ml, were incubated overnight at 4°C to ensure complete equilibration of the components of the resuspension media (17). On the next morning, the vesicles were divided into 25-μl aliquots suitable for individual uptake assays and frozen in liquid N₂ until the time of assay to ensure complete stabilization of the specific activity of substrate uptake over the course of an experiment (17). Under these conditions, very similar uptake rates were routinely obtained from the same batch of vesicles when the same conditions of uptake were employed in separate experiments on different days. Also, since large batches of vesicles tend to reduce variations in uptake data due to animal differences, quite comparable results were obtained between the two batches used in these studies.

**Transport Assays**—Initial rates of Na$^+$-dependent L-glutamic and D-aspartic acid uptakes were determined using the fast sampling, rapid filtration apparatus recently developed in our laboratory (18). For each assay, 20 μl of vesicles were loaded into the apparatus, and uptake was initiated by injecting the vesicles into 450 μl of the uptake media required for the particular assay (see the description in the figure and table legends). Tracer uptakes were determined at 35°C by a nine-point automatic sequential sampling of the uptake mixture at 1.5-s intervals. At each time point, the apparatus automatically injected 50 μl of the uptake mixture into 1 ml of ice-cold stop solution (see the description in the figure and table legends), filtered each stopped sample through 0.65-μm cellulose nitrate filters, and washed the filters three times with 1 ml of ice-cold stop. Radioactivities on the filters were then determined by liquid scintillation counting as described previously (19).

**Data Analysis**—Initial rates of [3H]L-glutamic acid and [3H]D-aspartic acid uptake were determined by linear regression over the nine-point time course of each assay, as described previously (18, 20). The kinetic parameters of acidic amino acid transport or inhibition were estimated by nonlinear regression analysis using the standard errors of regression on the initial rates as weighting factors (20). Curve fitting of various transport or inhibition models to the nontransformed data was performed after proper transformation of the corresponding rate equations as justified recently (21) and described previously (20). Both linear and nonlinear regression analyses were performed using the Enzfitter program (R. J. Leatherbarrow; 1987) and an IBM-compatible microcomputer. Only the best model fit to the data is reported in the figures, together with an Eadie-Hofstee transformation of the carrier-mediated process for visual appraisal of the goodness of fit (21).

**Chemicals and Protein Assay**—[3H]-D-aspartic acid (25 Ci/mmol) and [3H]-glutamic acid (25 Ci/mmol) were obtained from Amersham Corp. Unlabeled D-aspartic and L-glutamic acids were purchased from Aldrich.

Protein was measured with the BCA (bicinchoninic) assay kit from Pierce Chemical Co., using bovine serum albumin as a standard.

**RESULTS**

**Kinetics of L-Glutamic and D-Aspartic Acids at pH 6.0**—The initial rates of tracer L-glutamic acid and D-aspartic acid uptakes have been determined, as for all other experiments to be reported in this paper, under conditions of isotonicity and isoosmolarity and in the presence of an inwardly directed Na$^+$ gradient with the membrane potential clamped to 0 mV, using equal concentrations of the highly permeant anion iodide on both sides of the membrane (22).

A direct plot of the initial rates of [3H]-glutamic acid uptake at pH 6.0 as a function of varying concentrations of unlabeled L-glutamic acid in the uptake media is shown in Fig. 1. The best model fit to these data was that of two transport systems with high (Kₚₑ = 61 μM) and low (Kₚ₂ = 7.0 mM) affinities for the substrate working in parallel with diffusion. Fig. 1 (inset) shows that the curved nature of the Eadie-Hofstee transformation of the transport-mediated component is indeed compatible with the presence of more than one transport system and clearly demonstrates the goodness of fit.

Under the same assay conditions, Fig. 2 shows that the direct plot of the [3H]-aspartic acid data has a completely different shape when compared with Fig. 1. In fact, the initial rates of tracer uptake remained constant at D-aspartic acid concentrations of greater than 1 mM and actually matched the values of unspecific transport of L-glutamic acid. The best model fit to the data in that case was that of a single high affinity system (Kₚₐ = 80 μM) plus a diffusional component with no evidence to support more complex models of substrate uptake as shown by the Eadie-Hofstee plot in Fig. 2 (inset).

**Effect of D-Aspartic Acid on the Kinetics of L-Glutamic Acid at pH 6.0**—The good agreement between the Kₛ for D-aspartic acid transport and that for L-glutamic acid transport through the high affinity pathway seems to indicate that these two amino acids might share a common transport system. Accordingly, it should be possible to block [3H]-L-glutamic acid flux through the high affinity system by incorporating saturating concentrations of D-aspartic acid in the uptake media. The results of such an experiment are presented in Fig. 3, where the assay conditions were those of Fig. 1 but for the addition of AIB, methylaminoisobutyric acid.

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1 The abbreviations used are: Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; MeAIB, methylaminoisobutyric acid.

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**Fig. 1. Kinetics of L-glutamic acid at pH 6.0.** P¹ and P₂ were resuspended in 50 mM Mes-Tris buffer, pH 6.0, containing 156 mM choline iodide and 156 mM acetate-Tris, pH 6.0. Uptakes were performed in 50 mM Mes-Tris buffer, pH 6.0, containing 156 mM NaCl, 52 mM acetate-Tris, pH 6.0, 1.0 mM amiloride, 0.75 μM [3H]-glutamic acid, and 104 mM acetate-Tris plus L-glutamate-Tris, pH 6.0, with the indicated concentrations of unlabeled L-glutamic acid in each uptake media. The stop solution consisted of 50 mM Mes-Tris buffer, pH 6.0, containing 156 mM NaCl, 156 mM acetate-Tris, pH 6.0, and 0.05 mM MgCl₂.

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**Fig. 2. Effect of D-Aspartic Acid on the Kinetics of L-Glutamic Acid at pH 6.0.** The good agreement between the Kₛ for D-aspartic acid transport and that for L-glutamic acid transport through the high affinity pathway seems to indicate that these two amino acids might share a common transport system. Accordingly, it should be possible to block [3H]-L-glutamic acid flux through the high affinity system by incorporating saturating concentrations of D-aspartic acid in the uptake media. The results of such an experiment are presented in Fig. 3, where the assay conditions were those of Fig. 1 but for the addition...
Acid and L-Phenylalanine—Using the kinetic parameters the remainder should occur through the low affinity pathway that reached a maximum of
Fig. 2. Kinetics of D-aspartic acid at pH 6.0. The conditions were as described in the legend to Fig. 1, except that [3H]D-aspartic acid and unlabeled D-aspartic acid were used in the uptake media.

of 5 mM D-aspartic acid in the uptake media. Under these conditions, tracer uptake rates of L-glutamic acid remained constant at unlabeled substrate concentrations of less than 1 mM, and the best model fit to the data was that of a single low affinity system for L-glutamic acid uptake obtained in the absence of D-aspartic acid.

Inhibition of L-Glutamic Acid Uptake at pH 6.0 by D-Aspartic Acid and L-Phenylalanine—Using the kinetic parameters given in Fig. 1 and a substrate concentration of 50 μM, it can be calculated that about 20% of the total flux of L-glutamic acid should occur through the high affinity system, whereas the remainder should occur through the low affinity pathway and by diffusion across the membrane. As demonstrated in Fig. 4, increasing concentrations of D-aspartic acid in the uptake media caused an inhibition of L-glutamic acid uptake that reached a maximum of 25% of the rate obtained in the absence of inhibitor. Moreover, the estimated $K_i$ for competitive inhibition was 75 μM, in close agreement with the $K_m$ for D-aspartic acid transport at pH 6.0 (Fig. 2). On the other hand, increasing concentrations of L-glutamic acid progressively reduced the initial rates of D-aspartic acid uptake down to the level of diffusion, and the estimated $K_i$ for competitive inhibition was 129 μM (results not shown), in good agreement also with the $K_m$ for L-glutamic acid transport through the isolated high affinity pathway at pH 6.0 (Fig. 5). These results thus demonstrate quite clearly that D-aspartic and L-glutamic acids share a common high affinity transport route in the rabbit small intestine.

The nature of the low affinity pathway for L-glutamic acid transport cannot be inferred from these studies, however. Assuming that such a low affinity pathway could actually be represented by a neutral, ASC-like system in the brush border membrane, as has been found in other cell types (14–16), we thus tried to inhibit L-glutamic acid uptake by phenylalanine,
a neutral amino acid with large specificity for neutral amino acid carriers in the rabbit intestinal brush border membrane (23, 24). As shown in Fig. 4, addition of L-phenylalanine in the uptake media on top of saturating concentrations of D-aspartic acid caused a further inhibition of L-glutamic acid uptake rates down to the level of diffusion. The estimated $K_a$ for competitive inhibition by L-phenylalanine was 0.60 mM in that experiment.

If both L-glutamic acid and L-phenylalanine share the same transport system with low affinity for the former substrate, it should be possible to block [3H]L-glutamic acid flux through the low affinity pathway by incorporating saturating concentrations of L-phenylalanine in the uptake media. The results of such an experiment are presented in Fig. 5, where the assay conditions were those of Fig. 1 but for the addition of 50 mM L-phenylalanine in the uptake media. Under these conditions, tracer uptake rates of L-glutamic acid remained constant at unlabeled substrate concentrations in excess of 1 mM, and the best model fit to the data was that of a single high affinity system ($K_a = 87 \mu M$) plus a diffusional component, as was also obvious from the Eadie-Hofstee plot in Fig. 5 (inset). The $K_a$ of this system is identical with the $K_a$ of the high affinity pathway for L-glutamic acid transport obtained in the absence of L-phenylalanine (Fig. 1). However, the $V_{max}$ in the presence of L-phenylalanine was 8.85 $\pm$ 1.35 pmol/mg of protein/s, a value that represents an apparent 4.2-fold activation of the maximal velocity of the high affinity transport system.

**Effect of Acidic Amino Acids on L-Phenylalanine Uptake at pH 6.0 and 8.0**—Complete inhibition of the low affinity system for L-glutamic acid transport at pH 6.0 by L-phenylalanine implies a shared route for these amino acids under these conditions. If this were the case, it should be possible to inhibit L-phenylalanine uptake by incorporating excess concentrations of L-glutamic acid in the uptake media. Fig. 6 shows the effects of 100 mM L-glutamic acid or D-aspartic acid on the initial rates of L-phenylalanine uptake at pH 6.0 and 8.0. L-Glutamic acid caused a 70% inhibition of uptake at pH 6.0 and had a small but nonsignificant effect at pH 8.0. In contrast, D-aspartic acid had no effect on the initial rates of uptake at either pH.

**Effect of Amino Acids and Analogs on Uptake Rates of L-Glutamic and D-Aspartic Acids at pH 6.0**—In order to get a better appraisal as to the nature of the transport systems involved in the high and low affinity routes for L- acidic amino acid transport, we have studied the effect of different classes of amino acids and analogs on the initial uptake rates of both D-aspartic and L-glutamic acids. Tables I and II show the results of such an experiment using 50 mM concentrations of these agents in the uptake media in comparison to a control run in the presence of mannitol. Using D-aspartic acid (Table I), it appears that the acidic compounds, with the possible exception of D-glutamic acid, were all potent inhibitors, reducing uptake rates down to the level of diffusion obtained with 50 mM D-aspartic acid in the uptake media. None of the other compounds tested, however, could demonstrate any capacity to inhibit D-aspartic acid uptake. On the contrary, and quite interestingly in fact, it would appear that all of the neutral amino acids but glycine stimulated the uptake rates in excess of controls, thus suggesting a possible activation of the high affinity system by neutral amino acids. Both threonine and isoleucine proved the most efficient in this respect, with mean activations of 60% above controls.

With L-glutamic acid (Table II), the situation is more complex, but it is quite clear that the addition of D-aspartic, L-cysteinesulfenic, and L-cysteic acids to the uptake media caused about 30% inhibition of 50 mM L-glutamic acid uptake, in accordance with the flux expected through the high affinity system at pH 6.0 and this substrate concentration (Fig. 1). L-Aspartic acid, however, caused a 65% inhibition of uptake, thus suggesting an inhibition of both the high and low affinity transport systems.
Conditions for vesicle resuspension and uptake assays were the same as described in the legend to Table I, except that [3H]L-glutamic acid and cold glutamic acid were used in the uptake media.

Table II
Effect of amino acids and analogs on L-glutamic acid uptake rates at pH 6.0

| Test compounds | Initial rates | % of control |
|----------------|--------------|--------------|
|                | pmol/mg/sec  |              |
| Control mannitol | 4.97 ± 0.09  |              |
| Control L-glutamic acid | 1.04 ± 0.07  | 21           |
| L-Aspartic acid    | 1.78 ± 0.11  | 36           |
| D-Aspartic acid    | 3.42 ± 0.08  | 69           |
| D-Glutamic acid    | 4.09 ± 0.11  | 82           |
| L-Cysteine/ trifluoromethanesulfonic acid | 3.40 ± 0.09 | 68           |
| L-Cysteic acid     | 3.76 ± 0.27  | 76           |
| Glycine           | 3.49 ± 0.11  | 70           |
| L-Alanine         | 2.25 ± 0.25  | 45           |
| L-Valine          | 2.51 ± 0.08  | 51           |
| L-Leucine         | 2.68 ± 0.12  | 54           |
| L-Isoleucine      | 3.35 ± 0.13  | 67           |
| L-Serine          | 2.71 ± 0.11  | 55           |
| L-Threonine       | 3.56 ± 0.20  | 72           |
| L-Phenylalanine   | 3.39 ± 0.15  | 64           |
| L-Tryptophan      | 3.27 ± 0.25  | 66           |
| L-Histidine       | 2.98 ± 0.10  | 60           |
| L-Methionine      | 3.07 ± 0.21  | 62           |
| L-Arginine        | 4.43 ± 0.09  | 89           |
| Me-AIB            | 4.89 ± 0.22  | 98           |
| L-Proline         | 5.41 ± 0.27  | 109          |
| L-Lysine          | 4.54 ± 0.29  | 93           |

false initial rates of tracer uptakes (20, 21), as determined with the fast sampling, rapid filtration apparatus (18), we found evidence for both high and low affinity Na+-dependent transport systems for L-glutamic acid at pH 6.0 (Fig. 1). Under the same conditions, however, the uptake of D-aspartic acid was restricted to a single high affinity system (Fig. 2) that was limited by the high affinity transport system.

3.0 6.5 7.0 7.5

pH

Fig. 7. pH dependence of L-glutamic acid uptake. P1 and P2 were resuspended in 50 mM of either Mes-Tris (pH 6.0 and 6.5) or Hepes-Tris (pH 7.0, 7.5, and 8.0) buffers containing 156 mM choline iodide, 52 mM mannitol, and 5.2 mM acetate-Tris (pH adjusted). Uptakes were performed in the same buffers containing 156 mM Na+, 52 mM mannitol, or L-phenylalanine, 5.2 mM acetate-Tris or D-aspartate-Tris (pH adjusted), and 0.052 mM L-glutamic acid + 0.75 μM [3H]-L-glutamic acid. Triplicates were performed in each of the following uptake media: mannitol + acetate-Tris (total uptake), mannitol + D-aspartic acid (Asp-sensitive uptake), and L-phenylalanine + D-aspartic acid (diffusion). The points shown are the mean ± S.D. of the diffusion-corrected initial rates of transport. The D-aspartic acid-sensitive component of total transport is the difference between total and D-aspartic acid-insensitive transport rates.

DISCUSSION

Using stabilized and fully equilibrated preparations of rabbit jejunal brush border membrane vesicles (17) and following a protocol of nonlinear regression analysis of nontransformed true initial rates of tracer uptakes (20, 21), as determined with the fast sampling, rapid filtration apparatus (18), we found evidence for both high and low affinity Na+-dependent transport systems for L-glutamic acid at pH 6.0 (Fig. 1). Under the same conditions, however, the uptake of D-aspartic acid was restricted to a single high affinity system (Fig. 2) that was limited by the high affinity transport system.
The transport of 50 μM L-glutamic acid through the low affinity system X_{AG} in the intestines showed a pH optimum of 7.0 with declining rates of transport observed in shifting the pH toward 6.0 (Figs. 7 and 8). Bell-shaped pH-dependent profiles with L-glutamic acid as substrate, it would appear that this heterotropic activation occurs through a V_{max} effect only (Fig. 5). Such an activation of system X_{AG} by neutral amino acids has never been reported before in any cell type, and this property may well be specific to intestinal (and maybe renal) cells. It should be noted however that leucine has already been proposed as an allosteric modulator of the lysine transport system of the rat intestinal basolateral membrane (28). We are currently characterizing the effects of neutral amino acids on system X_{AG} in our vesicle preparation using D-aspartic acid as a specific substrate.

In conclusion, this study demonstrates that L-glutamic acid is transported by both high and low affinity Na^{+}-dependent transport systems at acidic pH, whereas D-aspartic acid serves as a specific substrate for the high affinity system. These findings bring to question many of the results obtained on the effects of varying pH and imposing H^{+} gradients across membranes using L-acidic amino acids as substrates, and they establish the necessity of using a specific substrate such as D-aspartic acid in future research on characterizing system X_{AG} in the intestinal epithelium.

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