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Characterization of a transport system for anionic amino acids in human fibroblast lysosomes

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L-Aspartate and L-glutamate are transported into human fibroblast lysosomes by a single, low K_m, Na^+-independent transport system, which has been provisionally named lysosomal system *d*. This system resembles the Na^+-dependent plasma membrane system x_{AG}^-, although these differences have been observed: (1) lysosomal system *d* recognizes the D- as well as the L-isomers of both aspartate and glutamate, whereas only for aspartate is the D-isomer recognized by system x_{AG}^-; (2) the anion L-homocysteate is not accepted by system x_{AG}^-, but is an effective inhibitor of lysosomal system *d*; (3) *N*-methyl, α-methyl, and ω-hydroxamate derivatives of both aspartate and glutamate inhibit lysosomal system *d*, but only the aspartate derivatives are accepted by system x_{AG}^-; (4) lysosomal system *d* shows a preference for the substrate amino group in the α-position, a preference not seen for system x_{AG}^-. These points imply differences at the two recognition sites with respect to substrate length, size and rotation, with the lysosomal site generally being the less restrictive.

Introduction

Lysosomes are a major site for macromolecule degradation in the cell. The degradation of proteins in lysosomes results in the accumulation of free amino acids, which exit the lysosomes through amino acid transport systems. Several lysosomal transport systems for amino acids have been described in a number of cell types, including two systems for large neutral and aromatic amino acids [1,2] one for small neutral amino acids [3], one for cationic amino acids [4,5], and one for cystine [6–9]. Most of these lysosomal transport systems are analogous to those amino acid transport systems found for the plasma membrane, although some distinct differences between the corresponding systems in the two locations have been observed (see Refs. 4,5, and reviews, Refs. 10 and 11).

Anionic amino acids are transported across the plasma membrane of mammalian cells by at least two systems. A Na^+-dependent system x_{AG}^- has been characterized in rat hepatocytes and human fibroblasts which transports L-aspartate and L-glutamate with approximately equal affinities [12–14]. The renal brush border also has a system superficially similar to system x_{AG}^- [15,16]. A Na^+-independent system x_C^- has been characterized in rat hepatocytes, rat hepatoma cells, and human fibroblasts which preferentially transports glutamate, homocysteate, and cystine [17–22]. The transport of aspartate and glutamate into human fibroblast lysosomes is mediated by a system similar to system x_{AG}^-, but which is less restrictive in certain aspects of substrate recognition. We report here the characterization of this lysosomal system, provisionally named lysosomal system *d*, with the provisional evocation, 'dicarboxylic'. Preliminary results on lysosomal system *d* have been reported [23].

Materials and Methods

Cell culture. Normal human fetal skin fibroblasts (GM0010) were obtained from the Human Genetic and Mutant Cell Repository. The cells were grown in 100-mm petri dishes or roller bottles in a 5% CO_2 atmosphere at 37°C in Coon's modification of Ham's F-12

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Abbreviations: Mops, 4-morpholinepropanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; PBS, phosphate-buffered saline; Hex, β-hexosaminidase.

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medium (Hazelton Research Products, Inc.), supplemented with 10% fetal calf serum (Hazelton).

Lysosomal preparation. Lysosomes were prepared as described previously [3]. Lysosomes were routinely resuspended in 100 mM citric acid buffered with 1 M Tris to pH 7.0.

Transport assays. For time courses, lysosomes were resuspended in 100 mM citrate/Tris buffer. Equal volumes of the ice-cold lysosome suspension and prewarmed L-[^{3}H]glutamate (20–40 Ci/mmol) or L-[^{3}H]aspartate (15–40 Ci/mmol) in 0.25 M sucrose were incubated at 37°C. At the indicated times, an aliquot was removed, added to 12 ml ice-cold phosphate-buffered saline (PBS), and filtered through a Whatman GF/A glass-fiber filter. The filter was quickly washed with two 12-ml washes of ice-cold PBS, then counted for radioactivity. Blanks were run using buffer containing no lysosomes. An aliquot of the lysosome suspension was used to determine lysosomal integrity by measuring latent β-hexosaminidase activity [4]. Only rough estimates of organelle water are possible at the present time, so the convention of expressing uptake data as pmol amino acid/unit latent β-hexosaminidase activity has been followed.

For kinetic and inhibition experiments, 15 μl of the ^{3}H-labelled amino acid in 0.25 M sucrose, and 15 μl of unlabelled amino acid or inhibitor in 50 mM citrate/Tris/0.125 M sucrose buffer (pH 7.0), were added together and prewarmed to 37°C. 15 μl of the ice-cold lysosome suspension in 100 mM citrate/Tris buffer was added with mixing, and 40 μl of the resulting suspension was removed at 1.5 min, which was then added to PBS, filtered, and washed as described above. Kinetic parameters were determined using a FORTRAN program [24] applying the Gauss-Newton non-linear least-squares method to the following equation

$$\log v = \log\{[(V_{max} \cdot S)/(K_m + S)] + K_d \cdot S\}$$

where K_d represents the estimated first-order non-saturable component of migration.

In experiments determining the effect of sodium ions on glutamate exodus, the lysosomes were preloaded with 2 mM L-[^{3}H]glutamate in 100 mM citrate/Tris buffer for 30 min at 37°C. They were then pelleted by centrifugation and resuspended in the selected prewarmed buffer. The β-hexosaminidase activity was determined promptly after the end of the experiment on the lysosomes remaining in the reaction mixture.

For *trans* experiments, lysosomes were pre-loaded in 50 mM citrate/Tris/0.125 M sucrose buffer containing 10 mM L-glutamic acid methyl ester for 20 min at 37°C, then pelleted, resuspended in 100 mM citrate/Tris buffer, and used as described above for time courses. For a control, the methyl ester was omitted.

Materials. All amino acids, inhibitors, and *p*-nitrophenyl-β-D-*N*-acetylglucosaminide were obtained from Sigma Chemical Co. Radioactive amino acids were obtained from Amersham. Percoll was purchased from Pharmacia P-L Biochemicals.

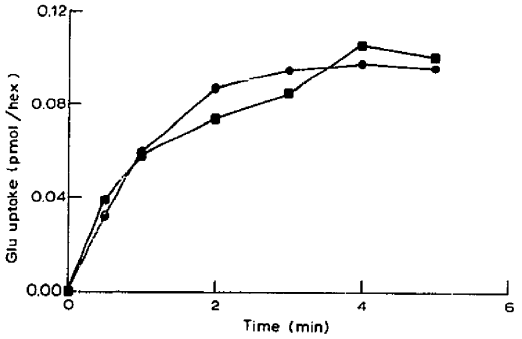

Fig. 1. Time course of L-glutamate uptake into human fibroblast lysosomes. Lysosomes were incubated with 10 μM L-[^{3}H]glutamate in 50 mM citrate/Tris buffer containing either 0.125 M sucrose or 0.077 M NaCl. An aliquot was removed at the indicated times and treated as described in Materials and Methods. ●, sucrose; ■, NaCl.

Results and Discussion

The time course of 10 μM glutamate uptake into lysosomes, in the presence of sucrose or NaCl, is shown in Fig. 1. Glutamate uptake appeared to be a Na^+-independent process. We also measured glutamate exodus from lysosomes in the presence of sucrose or NaCl. Extralysosomal Na^+ ions would alter the Na^+-ion gradient and would reduce the rate of glutamate exodus if exodus were dependent on the gradient. Fig. 2 shows the rate of glutamate exodus was not reduced. Both glutamate uptake and exodus, therefore, appeared to be Na^+-independent.

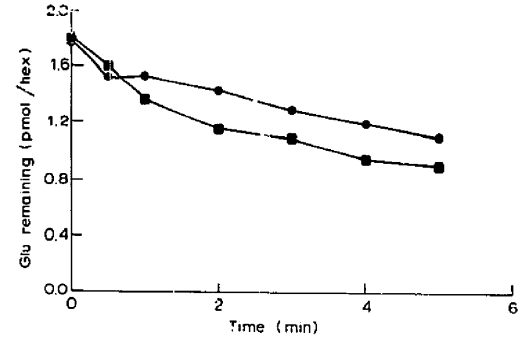

Fig. 2. Exodus of L-glutamate from human fibroblast lysosomes. Lysosomes were incubated with 2 mM L-[^{3}H]glutamate in 100 mM citrate/Tris buffer for 30 min, then pelleted. The lysosomes were then resuspended in 50 mM citrate/Tris buffer with either 0.125 M sucrose or 0.077 M NaCl. The radioactivity remaining in the lysosomes was determined at the indicated times. ●, sucrose; ■, NaCl.

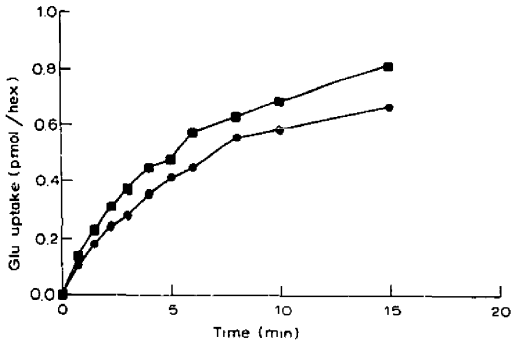

Fig. 3. *Trans*-effects on lysosomal glutamate uptake. Lysosomes were incubated with or without 10 mM glutamic acid methyl ester in 50 mM citrate/Tris/0.125 M sucrose for 20 min at 37°C, then pelleted. The lysosomes were then resuspended in 100 mM citrate/Tris buffer and incubated with 10 μM L-[^{3}H]glutamate in 0.25 M sucrose. At the indicated times an aliquot was removed and treated as described in Materials and Methods. ●, control; ■, preloaded.

The effect of intralysosomal glutamate on Na^+-independent glutamate uptake was determined by pre-loading the lysosomes with L-glutamate methyl ester before measuring the rate of uptake of 10 μM glutamate (Fig. 3). Glutamate uptake was not subject to strong *trans* effects.

The kinetic parameters of Na^+-independent glutamate uptake into lysosomes were determined using initial velocities of 1.5 min. Fig. 4a shows the Eadie-Scatchard analysis of these kinetic data. The apparent K_m values ranged between 4 μM and 12 μM over several experiments. The V_{max} values and the values representing the non-saturable component varied from experiment to experiment (probably because of variation in factors such as cell passage number and length of time at confluence). The linear plot is consistent with glutamate transport being mediated by a single, Na^+-independent system.

The kinetic parameters of lysosomal aspartate uptake were also determined. The Eadie-Scatchard plot of these data is shown in Fig. 4b. The apparent K_m values ranged between 4 μM and 12 μM over several experiments. The linear plot suggests that aspartate uptake is probably also mediated by a single Na^+-independent system.

Fig. 5a shows the double-reciprocal plot of aspartate inhibition of lysosomal glutamate uptake. The inhibition by aspartate appeared to be competitive, and the determined K_i value for inhibition was approx. 7 μM, in good agreement with the K_m value determined for aspartate uptake. Conversely, glutamate inhibition of lysosomal aspartate uptake appeared competitive (Fig. 5b), with agreement between the K_m value for glutamate uptake and the K_i value for glutamate inhibition (approx. 5 μM). Taken together, the kinetic data suggest that glutamate and aspartate are transported into lysosomes by a single Na^+-independent system, and that aspartate and glutamate have approximately equal affinities for this system. Lysosomal system *d* is, in this respect, most closely analogous to the plasma membrane system x_{AG}^-.

The effect of pH on Na^+-independent aspartate uptake into lysosomes was measured (Fig. 6). An approximate doubling of activity was seen between pH 5 and 8. The increase in activity cannot be attributable to an increase in the anionic form of aspartate, since most of the amino acid will already be in the anionic form at pH 5. The increase in activity may be the result of titration of a group on the transporter, making transport more efficient at higher pH, or of a pH-dependent change in some other parameter, such as an ion gradient, which then indirectly affects transport. The pH inside the lysosome has been estimated at approx. 5.3, whereas that of the cytoplasm is closer to 7.0 [25–27]. Under this pH gradient, net aspartate movement across the lysosomal membrane would be directed inward. The direction of net movement is determined, however, by

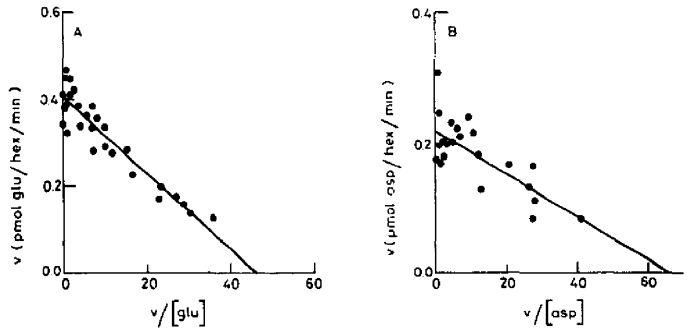

Fig. 4. Eadie-Scatchard plots of L-glutamate and L-aspartate uptake into human fibroblast lysosomes. (A) The uptake of L-glutamate at various concentrations was determined. A non-saturable component of 0.161 pmol/hex/min/mM was subtracted from each data point. $K_m = 9\ \mu M \pm 0.7$ for this experiment. (B) The uptake of L-aspartate at various concentrations was determined. A non-saturable component of 0.161 pmol/hex/min/mM was subtracted from each data point. $K_m = 3.4\ \mu M \pm 0.8$ for this experiment.

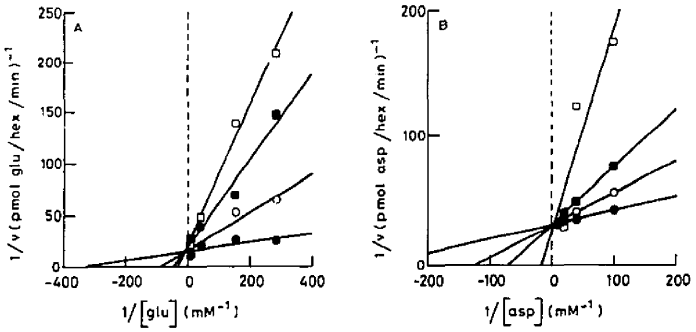

Fig. 5. Double-reciprocal plots demonstrating competitive inhibition between L-aspartate and L-glutamate for lysosomal transport. (A) Inhibition of L-glutamate uptake into human fibroblast lysosomes by L-aspartate. Aspartate concentrations were: ●, control (K_m for glutamate uptake = 3.6 μM ± 1.5); ○, 0.01 mM; ■, 0.05 mM; □, 0.10 mM. (B) Inhibition of L-aspartate uptake into human fibroblast lysosomes by L-glutamate. Glutamate concentrations were: ●, control (K_m for aspartate uptake = 3.8 μM ± 1.9); ○, 0.005 mM; ■, 0.015 mM; □, 0.050 mM.

several other factors, including the routes and rates of exodus and entry, and the concentrations of glutamate and aspartate in the lysosomes and the intracellular compartment [28,29].

We looked at a range of amino acids and amino acid analogs as inhibitors of Na^+-independent glutamate uptake to characterize the substrate specificity of the lysosomal anionic transport system (Table I). Aspartate, cysteine sulfinate, cysteate, homocysteate, and L-α-aminoadipate were very effective inhibitors. The longer D,L-α-aminopimelate showed less inhibition. Both the L-aspartate and the L-glutamate hydroxamate derivatives inhibited lysosomal glutamate uptake, the aspartate derivative being the more effective. In contrast, the glutamate hydroxamate derivative is ineffective as an inhibitor of system x^-_{AG} [14]. The chain length limitation for lysosomal system *d* is, therefore, slightly longer than that for system x^-_{AG}. Cystine, however, is too long, if not improperly charged, for both systems.

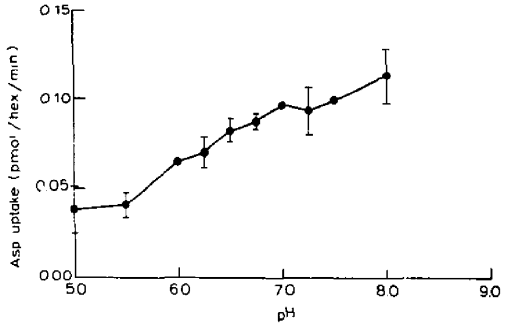

Fig. 6. The effect of pH on aspartate uptake into human fibroblast lysosomes. Lysosomes were resuspended in 0.25 M sucrose and incubated in 10 mM Mops (pH 5.0–6.5) or Mes (pH 6.5–8.0) buffers with 10 μM L-[^{3}H]aspartate and 0.25 M sucrose for 1.5 min at 37° C. An aliquot was then removed and treated as described in Materials and Methods. Points are the averages of three determinations. The error bars represent standard deviations.

Homocysteate, which has a bulky sulfonate group at the distal anion site, inhibited lysosomal system *d* but does not inhibit system x^-_{AG} [14]. This suggests that the lysosomal recognition site for the distal anion is less

TABLE I

Inhibition of glutamate uptake by amino acids and analogs

Glutamate uptake (10 μM) was determined in the presence of 10 mM inhibitor, unless otherwise indicated. The data are expressed as percentages of the uninhibited glutamate uptake rate. The values represent the averages of two determinations, with the ranges indicated in parentheses. The table combines the results of several experiments, in which control glutamate uptake values ranged between 0.06 and 1.4 pmol per unit latent hexosaminidase activity.

Inhibitor	% control
L-Aspartate	0
D-Aspartate	21 (20– 21)
L-Cysteinesulfinate	0
L-Cysteate	11 (9– 13)
L-Glutamate	0
D-Glutamate	33 (29– 36)
L-Homocysteate	21 (20– 21)
L-α-Aminoadipate	8 (8– 8)
D-α-Aminoadipate	67 (57– 77)
D,L-α-Aminopimelate	76 (71– 80)
L-Aspartate-β-hydroxamate	3 (2– 4)
L-Glutamate-γ-hydroxamate	29 (28– 29)
L-Cystine (0.2 mM)	107 (105–109)
N-Methyl-L-aspartate	51 (50– 52)
N-Methyl-D-aspartate	15 (15– 15)
N-Methyl-L-glutamate	64 (61– 68)
α-Methyl-D,L-aspartate	48 (47– 49)
α-Methyl-D,L-glutamate	34 (31– 36)
N-Acetyl-L-aspartate	27 (20– 35)
N-Acetyl-L-glutamate	105 (98–113)
3-Aminoglutarate	76 (72– 80)
α-L-Aspartyl-L-alanine	53 (52– 54)
β-L-Aspartyl-L-alanine	33 (32– 34)
Kainate	79 (78– 80)
L-Leucine	91 (80–103)
L-Serine	93 (89– 97)
2-Methylaminoisobutyric acid	122 (120–124)

restrictive than its plasma membrane counterpart. The *N*-methyl- and α-methyl-derivatives of both aspartate and glutamate inhibited lysosomal glutamate uptake by approx. 50%, whereas only the aspartate analogs are effective as inhibitors of system x^-_{AG} [14]. These findings suggest that the lysosomal recognition sites for the α-carbon and the α-amino group are also larger or more tolerant than the plasma membrane sites, although the longer glutamate cannot be accommodated when its amino group is acetylated.

The stereospecificity of lysosomal system *d* is also less restrictive than that seen for system x^-_{AG}. The L- and D-isomers of aspartate are transported by system x^-_{AG}, but only L-glutamate, and not D-glutamate, is accepted. In contrast, the D-isomers of both aspartate and glutamate inhibited lysosomal glutamate uptake, although they were not quite as effective as the L-isomers. These results imply that lysosomal system *d* can accommodate more variation than system x^-_{AG} in the length of the two groups on the α-carbon on either side of the α-amino group. There is a limit to this acceptance, since D-α-aminoadipate, with a longer butyrate group on one side of the α-carbon, has become a less effective inhibitor than L-α-aminoadipate.

3-Aminoglutarate, which has a β-amino group, hardly inhibited lysosomal glutamate uptake, although it is a strong inhibitor of system x^-_{AG}. The dipeptide β-L-Asp-L-Ala, although restricted in its acceptance by its length, is a better inhibitor of lysosomal system *d* than the dipeptide α-L-Asp-L-Ala. These results suggest that lysosomal system *d*, unlike system x^-_{AG}, has a preference for the substrate amino group in the α-position.

The cyclic glutamate analogue, kainate, which is used to discriminate excitatory amino acid receptors in the central nervous system [30,31], hardly inhibited glutamate uptake at all, in contrast to its effectiveness as an inhibitor for system x^-_{AG}. This was the only case observed in which lysosomal system *d* was the less tolerant system as to substrate size or conformation.

In summary, we have characterized a lysosomal transport system for anionic amino acids, provisionally named lysosomal system *d*. This system is similar to the plasma membrane system x^-_{AG} in that the natural substrates, L-aspartate and L-glutamate, appear to be transported by lysosomal system *d* with approximately equal and relatively high affinities. It is, however, less restrictive than system x^-_{AG} with respect to substrate length, size, and rotation. The plasma membrane system x^-_{AG} is sodium dependent, whereas the data presented here indicated that both uptake and exodus through lysosomal system *d* are sodium independent. There is, however, little information available about the distribution and flows of Na^+ ions across the lysosomal membrane, and technical difficulties in determining ion distribution prohibit us from exploring this aspect of lysosomal transport further at this time. A system analogous to system x^-_C has not been detected for the lysosome, although such a system would be pertinent to cystinosis [10,11].

Other characterized lysosomal transport systems also show features that distinguish them from the roughly corresponding amino acid transport systems of the plasma membrane [10,11]. The transport system defect in cystinosis is restricted to the lysosomes, since cystine transport at the plasma membrane is unaltered [32], suggesting, in this case, that the transporters at the two sites are genetically distinct. For most cases, however, the question as to whether the agencies for amino acid transport at the two locations are products of different genes or not remains unanswered.

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