Biochimica et Biophysica Acta 896 (1987) 247–255
Elsevier

BBA 72945

A novel imino-acid carrier in the enterocyte basolateral membrane

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(Received 19 August 1986)

Key words: Amino acid transport; Imino acid transport; Proline transport; Intestinal transport; Membrane transport; (Basolateral membrane vesicle)

Basolateral membrane vesicles prepared from rat small intestinal epithelial cells were used to study the sodium-independent transport of L-proline. The uptake of L-proline was unaffected by the presence of sodium and showed saturation kinetics (K_t = 0.5 mM and V_{max} = 23.3 pmol / mg protein per s). Competition experiments indicated that other amino acids had an affinity for the carrier system with L-leucine > L-alanine > sarcosine > glycine > L-lysine > OH-proline > taurine > β-alanine > D-alanine > D-proline > L-serine > phenylalanine > valine > D-serine > phenylalanine > valine > D-serine > MeAIB > methionine > threonine. This pathway does not resemble those previously described either in the brush-border membrane of intestinal epithelial cells or the plasma membrane of other cell types. The lack of effect of methionine and threonine indicate that proline is not using the L-type system, while the very low affinity for MeAIB and the Na^+ independence suggest that this is a novel system for imino acids. The relatively high capacity of this system and its low K_t, which is almost identical to the proline system in the brush-border membrane, strongly suggest that this is an important pathway in the final step for proline absorption by the small intestine.

Introduction

The sodium-dependent transport of proline and OH-proline across the intestinal brush-border membrane has been extensively studied in recent years, and although there appear to be some species differences, certain features are common [1–5]. Two major routes of entry for the imino acids predominate: one is the neutral amino-acid pathway shared with leucine, methionine, alanine, etc.; the other is a more specific imino-acid carrier. One particular feature shared by these imino-acid transporters is their high affinity for (methylamino)isobutyric acid (MeAIB). Also, the sodium-independent carrier-mediated uptake of proline across the brush-border membrane appears to be minimal, representing as little as 2.5% of the total uptake [6].

It must, however, be borne in mind that for transfer from the intestinal lumen to the blood stream to be completed, substrates must also cross the basolateral membrane. Recent reports have indicated the existance of several Na^+-independent transport mechanisms in the basolateral membrane, including an 'L-type' system carrying predominantly neutral amino acids like leucine, alanine and methionine [7], an Ly^+ system for dibasic amino acids [8] and several routes for cysteine [9]. However, the transport of imino acids across the basolateral membrane has received very little attention. Slack et al. [10] proposed that proline transport across this membrane was sodium-independent. The present study has characterized the sodium-independent transport of L-proline across the enterocyte basolateral mem-

Abbreviation: MeAIB, methylaminoisobutyric acid.

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brane using membrane vesicles. This transport system possesses some unique features not previously described for an imino-acid carrier, in particular its very low affinity for MeAIB.

Materials and Methods

Basolateral vesicles were prepared from rat intestinal mucosal scrapings using a modification of the technique of Scalera et al. [11]. Jejunal or ileal segments of the small intestine were removed from 2–4 male Sprague-Dawley rats (300–350 g) and the lumen was rinsed with 10 ml ice-cold phosphate-buffered saline (pH 7.4). Mucosal scrapings were taken with a glass slide and placed in 60 ml ice-cold membrane suspension solution (250 mM sucrose/2 mM Tris-HCl (pH 7.4)/0.1 mM phenylmethylsulfonyl fluoride). The suspended scrapings were homogenized using a Polytron homogenizer (4×30 s pulses) and centrifuged at $2500 \times g$ for 15 min. The resultant supernatant was removed and centrifuged at $20\,500 \times g$ for 20 min. This centrifugation produced a double pellet consisting of a hard brown lower pellet and a fluffy white upper pellet. The fluffy upper pellet was isolated by pouring off the supernatant, adding 10 ml of membrane suspension solution and gently swirling the centrifuge tube such that only the fluffy pellet was dislodged.

To purify basolateral vesicles, the resuspended fluffy pellet was subjected to glass-Teflon homogenization (10 strokes; 1500 rpm) mixed with Percoll and membrane suspension solution (final volume 35 ml; 12% (v/v) Percoll) and centrifuged at $48\,000 \times g$ for 1 h to obtain a density gradient. The gradient was separated from top to bottom into 19 fractions and fractions 5, 6 and 7 were pooled as basolateral vesicles. The vesicle suspension was diluted with membrane suspension solution to a volume of 35 ml and centrifuged at $48\,000 \times g$ for 30 min to obtain the basolateral vesicle pellet. The basolateral vesicle pellets were resuspended in the media required for the particular experiment, assayed for protein, and diluted as required.

The purity and recovery of the basolateral membrane were determined by measuring marker enzyme activities. Alkaline phosphatase and ouabain-sensitive $(Na^+ + K^+)$-ATPase were assayed according to Parkinson et al. [12]. Cytochrome *c* oxidase was assayed following the procedure of Cooperstein and Lazarow [13]. NADPH:cytochrome *c* reductase was assayed by the method of Sottocasa et al. [14]. Protein was measured using the technique of Bradford [15].

The uptake of the proline into vesicles was initiated by mixing a volume of vesicles with an equal volume of uptake media containing L-proline + 100 μCi L-[5-^{3}H]proline/mol L-proline). 40-μl samples were extracted from the mixture at the desired times and diluted with 1.125 ml of ice-cold stop solution (125 mM NaCl/2 mM Tris-HCl (pH 7.4)/0.1 mM $HgCl_2$), and 1.0 ml of the diluted mixture was transferred to a cellulose acetate filter (0.45 μm pore size). The filter was washed with 5 ml ice-cold stop solution then placed in scintillation vials for liquid scintillation counting. Proline actually held by the vesicles was calculated by correcting for L-[5-^{3}H]proline retained on the filter in the absence of vesicles.

For determining proline uptakes using short incubation periods (i.e., 10 s or less), a 20 μl drop of vesicles and a 20 μl drop of uptake media were placed in close proximity on the bottom of a polycarbonate tube. Uptake was initiated by vortexing the tube and at the conclusion of the uptake period 1.125 ml of ice-cold stop solution was rapidly injected into the mixture. Proline held by the vesicles was determined as previously described.

Efflux experiments were conducted using vesicles which had been preloaded at 4°C overnight. The preloading solution contained the required proline concentration, radiolabelled proline and sorbitol at a concentration suitable to maintain the osmolarity of the solution. 20-μl aliquots of vesicle solution were subsequently incubated at room temperature for 10 s in substate-free medium (125 mM NaCl/2 mM Tris-HCl (pH 7.4)) and filtered. Proline remaining in the vesicles was measured as described above. The 'zero-time' content of the vesicles was measured by performing the identical procedure at 4°C. The difference between the two was taken as the 10 s efflux rate.

L-[5-^{3}H]Proline was obtained from Amersham International, U.K. All amino acids were from Sigma Chemical Company and 0.45 μm cellulose acetate filters were supplied by MSI, Honeoye Falls, NY.

Results

Purification of basolateral vesicles and proline binding

Marker enzyme analysis of the pooled membrane fraction taken off the Percoll gradient was compared with the specific activity of the initial mucosal homogenate. Alkaline phosphatase showed an enrichment factor of 0.1 (0.9 vs. 11.3 μmol *o*-nitrophenol produced/h per mg protein). Cytochrome *c* oxidase had an enrichment factor of 0.02 (2.1 vs. 85.0 μmol cytochrome *c* oxidase/h per mg protein). Similarly NAPDH cytochrome *c* reductase specific activity was also reduced this time by a factor of 0.01 (0.03 vs. 3.47 mol cytochrome *c* reduced/h per mg protein). In contrast, ouabain-sensitive $(Na^+ + K^+)$-ATPase activity was enriched 18.0-fold in this preparation compared to the original mucosal scrapings (194.8 vs. 10.8 μmol PO_4 produced/h per mg protein). These data show a considerable degree of purification of these membranes with little contamination of either intracellular or brush-border membranes.

Binding of L-proline to the membrane vesicles was estimated by allowing vesicles to come to equilibrium for 20 min with 1 mM L-proline in media of increasing osmolarity. The data shown in Fig. 1 indicate that with a decreasing ratio of osmolarity inside/osmolarity outside the vesicles shrank and contained less proline. However extrapolation to a zero intercept (infinite osmolarity outside the vesicles) gave a *y* intercept significantly different from zero (equivalent to 18% total proline). Hence 18% of the proline associated with the vesicles was not inside an osmotically active space but bound to the membranes. Subsequent uptake data were corrected for binding as described below.

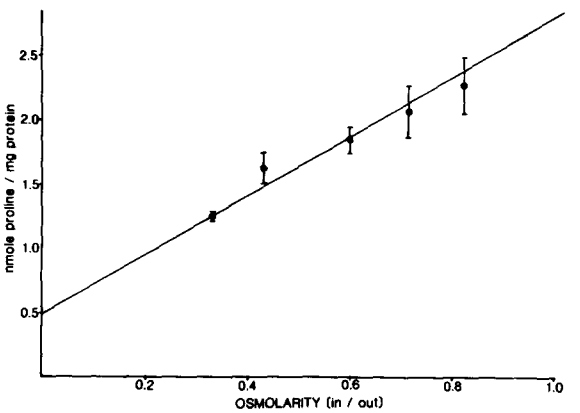

Fig. 1. Effect of medium osmolarity on the equilibrated proline content of jejunal basolateral vesicles. Basolateral vesicles were prepared as described in Materials and Methods and the final vesicle pellets were resuspended in 250 mM mannitol/2 mM Tris-HCl (pH 7.4) to give a concentration of 2.82 mg vesicle protein/ml and allowed to equilibrate overnight. The vesicles were then divided into five fractions. Proline uptake was initiated by mixing the fraction with an equal volume of uptake media containing 250 mM mannitol, 1 mM Tris-HCl (pH 7.4), 2 mM L-proline + [^{3}H]proline, and varying concentrations of cellobiose to give the indicated ratio of osmolarity in/osmolarity outside the vesicles. At 20 min from initiation of uptake, samples were taken to assay vesicle proline content using the microporous filtration technique. Each point represents the mean proline content ± S.E. The *y*-intercept of the regression line is significantly greater than 0 ($P = 0.05$) and indicates that proline uptake is composed of 82% intravesicular accumulation and 18% binding to the vesicles.

Vesicle sidedness

The orientation of the vesicles has been in-

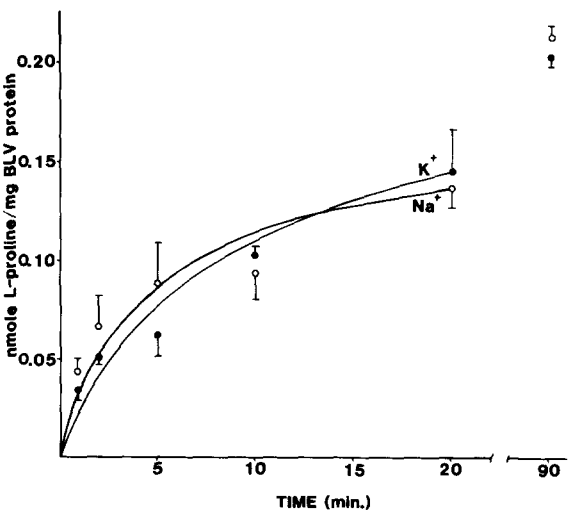

Fig. 2. Time-course for L-proline uptake across basolateral membranes in the presence of NaSCN and KSCN gradients. Vesicles were prepared and resuspended as described in Fig. 1. Proline uptake was initiated (time zero) by mixing a volume of vesicle suspension with an equal volume of uptake media (250 mM mannitol 2 mM Tris-HCl (pH 7.4), 1.0 mM L-proline + [^{3}H]proline, 200 mM NaSCN or 200 mM KSCN) to give a final concentration of 0.5 mM L-proline. Uptakes were performed in triplicate and intravesicular proline content was calculated by correcting for the binding component of total uptake. The points represent the mean intravesicular proline content at the indicated time from initiation of uptake.

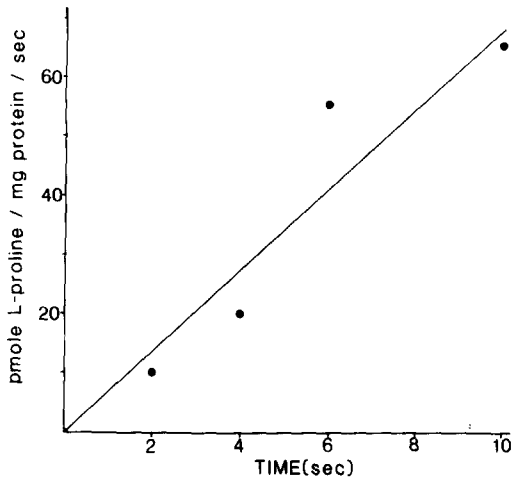

Fig. 3. Initial rate of proline equilibration across basolateral membranes. The vesicles were resuspended in 125 mM NaSCN/2 mM Tris-HCl (pH 7.4) to give a concentration of 2.82 mg vesicle protein/ml and allowed to equilibrate overnight to give a final proline concentration of 0.5 mM. Proline uptakes were initiated by mixing a 20 μl drop of vesicles with a 20 μl drop of uptake medium (125 mM NaSCN/2 mM Tris-HCl (pH 7.4)/1.0 mM L-proline + L-[^{3}H]proline). The drops were placed in close proximity on the bottom of a polycarbonate tube and uptakes were performed as described in Materials and Methods. The data were corrected for binding and each point represents the mean value obtained from three determinations of intravesicular proline content at the indicated time from initiation of uptake.

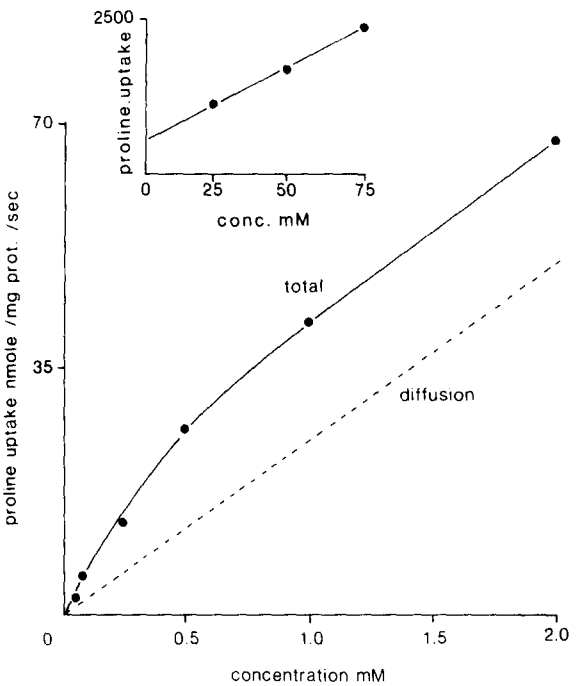

Fig. 4. Effect of varying concentrations of proline on the initial rate of proline equilibration across basolateral membranes. Vesicles were prepared and uptake was performed as described in Fig. 3. The uptake media contained varying concentrations of L-proline plus mannitol which, in all cases, equalled 150 mM. Initial rates of proline uptake were determined using 5-s incubations and the points represent the mean values obtained from triplicate assays at each of the indicated concentrations of proline.

vestigated by looking at the effect of trypsin and chymotrypsin on the transport of D-glucose. Measurements made with isolated enterocytes have, shown that the glucose transporter in the basolateral membrane has an exofacial site which is sensitive to chymotrypsin but only slightly so to trypsin. Similarly, several separate vesicle preparations have shown considerable sensitivity to chymotrypsin but at the most 20% susceptibility to trypsin (Maenz and Cheeseman, unpublished data). This strongly suggests that the vesicles are at least 80% outside out, with the remaining 20% inside out.

Time-course and sodium dependence of L-proline uptake

L-Proline was taken up into the basolateral membrane vesicles in a time-dependent manner with a half-time for equilibration of about 2 min (Fig. 2). There was no evidence of an overshoot in the presence of sodium, and replacement of NaSCN with KSCN in the incubation medium had no significant effect on the time-course of L-proline uptake. Also the equilibrium space was not affected by the replacement of sodium with potassium. In order to ascertain accurately the concentration dependence of proline uptake it was necessary to measure initial rates of uptake; consequently the time-course over the first 10 s was determined (Fig. 3). Clearly, the uptake of L-proline is linear up to 10 s, and so in all subsequent experiments 5 s incubations were employed.

Concentration dependence of L-proline uptake

To assess whether a component of the proline uptake into the vesicles was carrier-mediated or just simple diffusion, the kinetics of the process were studied.

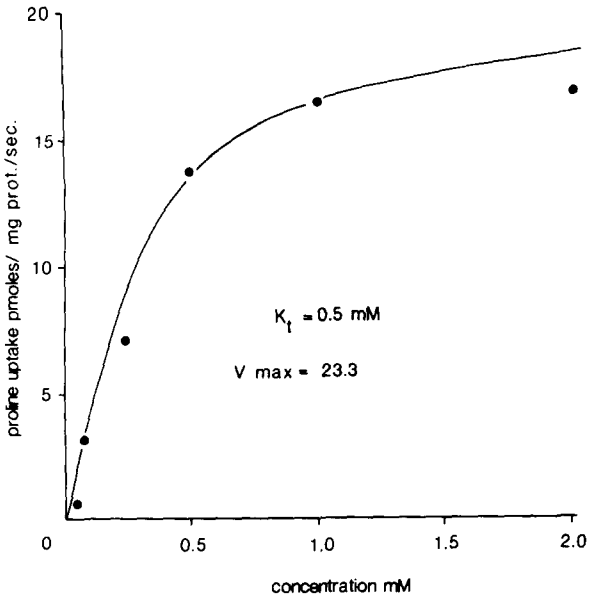

Fig. 5. Initial rates of carrier-mediated proline uptake across basolateral membranes. The linear portin of the curve in Fig. 5 was used to estimate the nonsaturable component (i.e., binding plus diffusion) of total proline uptake. The data were then corrected for nonsaturable uptake to determine the initial rates of carrier-mediated proline uptake.

Proline uptake rates over the concentration range of 60 μM to 75 mM were characteristic of diffusion plus carrier-mediated transport (Fig. 4). At the higher concentrations of 25, 50 and 75 mM proline, carrier-mediated transport appeared to be saturated, resulting in a linear relationship between proline concentration and initial rates. The slope of this linear component of the graph was used to calculate the diffusion constant for proline uptake into the vesicles. Uptake at the lower substrate concentrations was then corrected for diffusion by subtracting the calculated non-carrier mediated component for each concentration to yield a net carrier-mediated uptake shown in Fig. 5, which using an Eadie-Hofstee transformation gives a K_t of 0.5 mM and a maximal velocity of 23.3 pmol/mg protein per s. The binding of the L-proline to the membranes was assumed to be a component of the linear uptake observed at high substrate concentrations. Consequently, no further correction was employed.

TABLE I

EFFECT OF POTENTIAL INHIBITORS ON THE INITIAL RATE OF CARRIER-MEDIATED L-PROLINE UPTAKE ACROSS BASOLATERAL MEMBRANES

Basolateral vesicles were prepared and proline uptake experiments were performed as described in Fig. 3. The final concentration of inhibitor was 50 mM. Data was corrected for binding and diffusion using the coefficient calculated from the uptake at high proline concentrations. Values are the mean ± S.E. of triplicate determinations when expressed as % inhibition of the initial rate of carrier-mediated L-proline uptake obtained in the presence of 50 mM mannitol.

Inhibitor	Inhibition of proline (%)
L-Proline	100.0 ± 29.9
L-Leucine	113.1 ± 26.4
L-Alanine	108.7 ± 5.2
Sarcosine	94.7 ± 2.7
Glycine	94.2 ± 59.7
L-Lysine	90.9 ± 9.4
OH-Proline	90.8 ± 13.9
Taurine	81.7 ± 23.1
β-Alanine	77.8 ± 33.0
D-proline	69.4 ± 16.4
L-Serine	68.6 ± 18.5
Phenylalanine	68.4 ± 8.5
Valine	58.4 ± 1.7
D-Serine	54.7 ± 14.1
MeAIB	53.0 ± 15.0
Methionine	38.3 ± 1.2
Threonine	−25.4 ± 56.3

Concentration dependence of L-proline efflux

As for uptake, the efflux data were also characteristic of diffusion plus carrier-mediated transport (data not shown). However, kinetic analysis indicated a possible asymmetry to the system. The K_t for the efflux was 2.4 ± 0.4 mM, while the V_{max} was 309 ± 241 pmol/mg protein per s.

Specificity of proline transport system

Table I shows the effect of 50 mM amino acids on 0.5 mM L-proline uptake. Two features indicate that the carrier exhibits stereospecificity. D-Proline has a much smaller inhibitory effect than L-proline or OH-proline and L-alanine apparently has a much higher inhibitory effect than either D- or β-alanine. The neutral acids L-leucine and L-alanine were very effective in inhibiting transport and so was the dibasic amino acid, L-lysine.

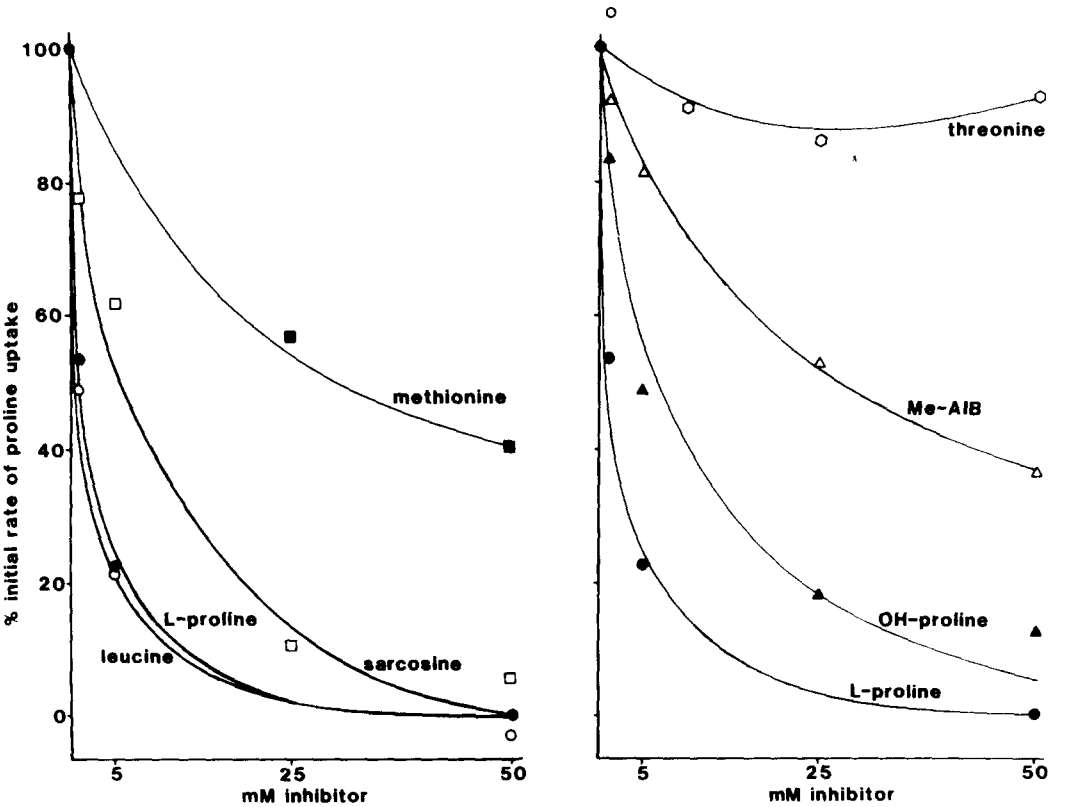

Fig. 6. Effect of varying concentrations of selected inhibitors on the initial rate of carrier-mediated L-proline uptake across basolateral membranes. Vesicle preparations and proline uptakes were performed as described in Fig. 3. The uptake media contained 0.10 mM L-proline + [^{3}H]proline, and varying concentrations of inhibitor plus mannitol equalling 100 mM. Data were corrected for binding plus diffusion and the points represent the mean of triplicate determinations when expressed as a percentage of initial rate of carrier-mediated proline uptake in the absence of inhibitor.

Glycine appears to be an effective inhibitor, although there was considerable variation between experiments. The closely related *N*-methylglycine (sarcosine) was also a good inhibitor and these observations were far more reproducible.

Several amino acids, including MeAIB and L-methionine, had a very small inhibitory effect, while threonine had no effect at all.

Competitive inhibition

Six of these amino acids were selected for a more detailed study of the kinetics of inhibition. In each case a range of concentrations of unlabelled amino acid was employed to inhibit the uptake of 50 μM and 100 μM L-proline. The results for 50 μM L-proline are shown in Fig. 6 and in each case inhibition appeared to be competitive and involve only one transporter for proline. These data confirmed the initial observations made with the single high concentration of inhibitor and 0.5 mM proline. Leucine, sarcosine and OH-proline were very effective inhibitors, while

TABLE II

K_i VALUES CALCULATED FROM THE DATA PRESENTED IN FIG. 7

Inhibitor	K_i (mM)
Leucine	0.44
Proline	1.88
OH-Proline	3.89
Sarcosine	0.74
Methionine	66.07
MeAIB	50.26

methionine and MeAIB had a limited effect and threonine no significant effect at all. By plotting $1/v$ against the inhibitor concentration, [I], for the two concentrations of substrate, the K_i for the inhibitor can be estimated from the point at which the two lines intersect. At that point

$$[S]_i(K_i + [I]) = (K_i + [I])[S]_2$$

but

$$[S]_i \neq [S]_2$$

therefore

$$[I] = -K_i$$

Fig. 7 shows these curves for the six inhibitors and Table II summarises the K_i values which were found. Sarcosine and L-leucine have the same affinity for the carrier as L-proline, while OH-proline appears to be able to bind less tightly, assuming that binding to the carrier is the rate-limiting step in overall translocation. Methionine and MeAIB have very low affinity and threonine has no detectable interaction with this system.

Discussion

These data strongly support the hypothesis that there is carrier-mediated transport of L-proline across the enterocyte basolateral membrane. When

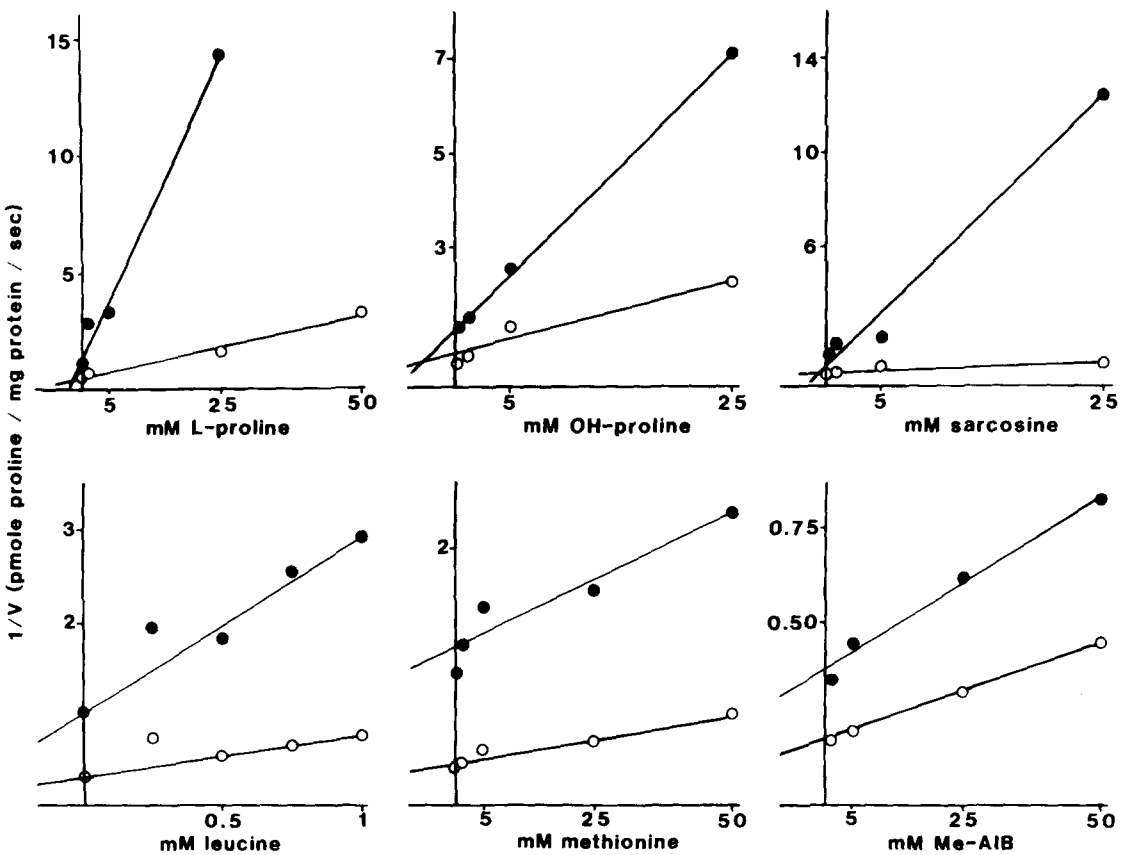

Fig. 7. Effect of varying concentrations of selected inhibitors on the initial rate of carrier-mediated uptake of 50 μM (○) and 100 μM (●) [^{3}H]proline. Procedures were the same as described in Fig. 6 except that uptakes were carried out using a final concentration of 50 μM or 100 μM L-proline + [^{3}H]proline. The specific activity of the uptake media was the same in all cases. The points represent the mean of triplicate determinations of 1/initial rate of carrier-mediated L-proline uptake at each of the indicated concentrations of inhibitor.

total unidirectinal fluxes are corrected for binding to the membranes and the non-saturable component, there remains a saturable component with a K_t of 0.5 mM and a V_{max} of 23.3 pmol/mg protein per s. These data are similar to those obtained by Mircheff et al. [7] for sodium-independent alanine transport across the basolateral membrane. Their data also showed non-saturable and saturable uptake of alanine into basolateral vesicles which they concluded represented both passive diffusion and carrier-mediated uptake.

Our competititon data also support the view that the saturable component of proline uptake is carrier-mediated. Inhibition is stereospecific, D-proline is far less effective than L- or OH-proline, while several amino acids have no effect at all and sarcosine and L-leucine can abolish proline transport.

It could be argued that proline is being transported by a previously described carrier system, i.e., the 'L-type' system [4,16] or those for cysteine [9]. Several amino acids which use the 'L-type' system inhibited proline uptake, namely L-leucine, alanine and glycine. However methionine, threonine and phenylalanine which have K_i values of 1.25, 0.44 and 0.59 mM, respectively, for the L-type system [7] had little or no effect on proline transport across the basolateral membrane. These data appear to exclude the possibility of proline using the 'L-type' system.

The pattern of inhibition also rules out the three routes for cysteine transport as mediators for proline uptake. None of these systems showed the same pattern of specificity found for proline, namely a high affinity for leucine, alanine, sarcosine and glycine while failing to react with threonine or only slightly with methionine [9]. Finally, although lysine does inhibit proline uptake there is no evidence for proline being able to affect lysine transport [8], and the unique feature of the Ly^+ system being stimulated by neutral amino acids indicates that the proline route is a separate one.

Comparison between the specificity of this proline transport system and the one in the brush-border membrane is of interest for several reasons. The first obvious difference is the lack of sodium-dependence in the basolateral membrane, Stevens et al. [6] found that in the rabbit brush-border membrane only 5% of the total uptake of proline (50 μM) was sodium-independent. Half of that flux was considered to be diffusion and 2.5% was inhibited by alanine, indicating that it went via the 'L-type' carrier. Secondly, although the basolateral and brush-border membrane proline systems appear to have several common structural requirements for transport, there are some important differences. MeAIB is often considered a model substrate for Na^+-dependent proline transporters [17,18], yet this amino acid has a very low affinity for the basolateral membrane transporter: the K_i is 68 mM. Conversely, sarcosine has a very high affinity for the basolateral membrane system ($K_i = 0.6$ mM), whereas the K_i for the *N*-methyl derivative of glycine is 2–20 mM for the brush-border membrane carrier [18].

The overall specificity of this proline carrier in the rat basolateral membrane appears to show a marked parallel with the sodium-dependent β-alanine system described for the brush-border membrane of the rabbit ileum. Both appear to have a high affinity for proline, OH-proline leucine, lysine and alanine and a low affinity for MeAIB. However the β-alanine carrier apparently cannot accept any methylated amino acids, whereas this basolateral membrane iminoacid system has a very high affinity for sarcosine (*N*-methylglycine).

The apparent kinetic asymmetry of this system, with its higher K_t and V_{max} at the inward-facing site, would make this transporter an ideal system for promoting efflux of imino acids from the enterocyte towards the blood stream.

These observations raise the number relatively high capacity Na^+-independent amino-acid transporters which have been characterised in the intestinal basolateral membrane to at least four. Of these only the 'L-type' system appears to be very similar to carriers described for other cell types, while the imino acid and the lysine carriers appear to have features unique to the intestine.

Acknowledgements

This work was supported by the Canadian MRC. S.D. was supported by an AHFMR summer studentship. D.D.M. is an AHFMR postdoctoral fellow.

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