SUBSTRATE STRUCTURE AND AMINO ACID/K+ SYMPORT IN BRUSH-BORDER MEMBRANE VESICLES FROM LARVAL MANDELA SEXTA MIDGUT

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Summary
The effects of amino acid sidechain length, substituent position and chirality on amino acid/K⁺ symport have been examined in rapid filtration experiments on brush-border membrane vesicles prepared from larval Manduca sexta midgut. Cis-inhibition and trans-stimulation protocols were used to examine the effects of amino acid analogs on the uptake of alanine, phenylalanine, leucine and lysine, which are cotransported with K⁺ by a zwitterionic symporter at the high pH characteristic of the midgut in vivo. The symporter was found to translocate both L- and D-stereoisomers of alanine, leucine and lysine, but only the L-form of phenylalanine. Alterations to substrate structure that leave the charge distribution unchanged do not affect symport. Thus, moving the methyl group from C-3 to C-5 in the sequence isoleucine, leucine and norleucine has no effect on their ability to inhibit leucine symport. Increasing sidechain length among alanine homologs has little effect on their ability to inhibit alanine uptake, but increasing the sidechain length of lysine homologs from 1 to 3 methylene groups enhances cis-inhibition and trans-stimulation of lysine symport. The substantial difference in molecular charge distribution among aminobutanoic acid isomers has a large impact on alanine symport with only α- (or 2-) aminobutanoic acid functioning as an alanine analog. Only those changes in substrate structure that are coupled to the molecular charge distribution seem to affect symport. The tolerance of the symporter may reflect a balance mandated by the conflicting demands of selectivity and throughput.

Introduction
Amino acid/cation symport is the coupling of transmembrane fluxes of an amino acid and a cation via a symporter protein. Symport is driven in vivo by the electrochemical gradient of either of the two substrates. Symporters are highly concentrated in the apical membranes of columnar cells, the brush-border membrane, in lepidopteran midgut (Giordana et al. 1989) where they mediate a massive uptake of amino acids used for growth and energy (Parenti et al. 1985). The symported cation is usually K⁺, the driving

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force is mainly electrical, and amino acids that are substrates for various putative symporters have been identified. However, the structural features that enable an amino acid to function as a symporter substrate have generally not been identified.

$K^+$ or $Na^+$ is required for amino acid transport into brush-border membrane vesicles (BBMVs) from the midgut of *Philosamia cynthia* at pH 7.4 (Hanozet *et al.* 1980) and *Manduca sexta* at pH 8.0 (Hennigan and Wolfersberger, 1989). $K^+$ selectivity over $Na^+$ is greatly enhanced at the more nearly physiological pH of 10 for the ‘neutral’ amino acids (i.e. those that are zwitterionic at neutral pH) alanine and phenylalanine (Hennigan *et al.* 1993a,b) and for the basic amino acids lysine, arginine and histidine (Z. Liu, unpublished data). This preference for $K^+$ is expected since $K^+$ is the major cation in the lumen (Harvey *et al.* 1975) and is required for transepithelial transport (Nedergaard, 1972).

The driving force for amino acid symport across brush-border membranes in vivo is a $>240 \text{ mV}$ (lumen positive) electrical potential difference ($\Delta V$) (Giordana *et al.* 1989; Wood *et al.* 1969; Moffet and Koch, 1988; Dow and Peacock, 1989), there being no $K^+$ activity gradient (Dow *et al.* 1984; Dow and Harvey, 1988). The $\Delta V$, which is imposed by a V-ATPase (Schweikl *et al.* 1989) in parallel with a $K^+/H^+$ antiporter (Wieczorek *et al.* 1991), not only drives symport but also renders the lumen highly alkaline (Dow, 1984, 1992; Chao *et al.* 1991; Harvey, 1992).

At least six group-specific amino acid symporters have been postulated at near-neutral pH in *P. cynthia* BBMVs (Giordana *et al.* 1989), including (a) a zwitterionic symporter, (b) a lysine symporter and (c) a glutamate symporter. All of the neutral amino acids as well as lysine use the zwitterionic symporter in *M. sexta* BBMVs at pH 10 (Hennigan *et al.* 1993a,b).

Most studies of amino acid uptake by lepidopteran midgut vesicles have focused on symport kinetics (Parenti *et al.* 1992), on ion specificity (Hennigan *et al.* 1993b) or on defining amino acid cohorts that share the same pathway (e.g. Giordana *et al.* 1989). In this report, we explore the connection between amino acid structure and symport with experiments on the uptake of representative labeled amino acids in the presence of analogs that differ in a specific structural feature from the labeled amino acid. This approach straightforwardly delineates the structural tolerance of the physiologically important symporter used by the labeled substrate. The use of amino acid analogs to help characterize transport systems is well-established in studies on whole cells (Oxender and Christensen, 1963; Daniels *et al.* 1969; Preston *et al.* 1974; Christensen, 1984, 1985). The present study extends structure–function correlations to vesicles for the first time. Cis-inhibition without a salt gradient and trans-stimulation (countertransport) of $K^+$-dependent uptake of labeled neutral and basic amino acids by analogs differing in length, chirality, substituent position and aromaticity have been examined at high pH.

### Materials and methods

BBMVs were prepared from the midguts of fifth-instar *M. sexta* larvae by Mg$^{2+}$ precipitation and differential centrifugation as described previously (Biber *et al.* 1981; Eisen *et al.* 1989). Enrichment factors for marker enzymes were reported by Eisen *et al.* (1989). Vesicles were loaded with buffers, whose compositions are specified in figure
legends, by suspension in the appropriate medium for 1 h on ice, centrifugation at 30900 g for 30 min at 4 °C and resuspension of the resulting pellet to the desired concentration (4–7 mg protein ml\(^{-1}\)). Typically, experiments were conducted on vesicles stored on ice overnight, but vesicles used immediately after preparation yielded very similar results. The vesicles formed by this process are osmotically sealed. Above-equilibrium accumulations of leucine, alanine and phenylalanine (Hennigan et al. 1993b), as well as lysine (Fig. 1), were found only in the presence of a salt gradient, confirming the presence of symport. However, all of the experiments with analogs reported in this paper were conducted in the absence of salt gradients so as to simplify interpretation of the results. The analogs used and their pK values are listed in Table 1.

Our rapid filtration protocols adhered closely to those used in prior work (Hennigan et al. 1993a,b). In inhibition experiments, samples of vesicles treated with valinomycin at 8 µg mg\(^{-1}\) protein (Hennigan et al. 1993b), and equilibrated with 50 mmol l\(^{-1}\) aminomethylpropanediol (AMPD) acidified to pH 10 with HCl, 100 mmol l\(^{-1}\) mannitol and 100 mmol l\(^{-1}\) KSCN, were incubated with an equal volume of transport buffer: 50 mmol l\(^{-1}\) AMPD, 100 mmol l\(^{-1}\) mannitol, 100 mmol l\(^{-1}\) KSCN, 1 mmol l\(^{-1}\) labelled substrate and 20 mmol l\(^{-1}\) test inhibitor. Valinomycin was added from a stock solution in ethanol 30 min before commencing the experiments. The concentration of ethanol so introduced was less than 1%. After a preset period, transport was terminated by the addition of 4 ml of ice-cold stop solution (transport buffer lacking substrate) and the vesicles were rapidly filtered through a nitrocellulose filter (Sartorius, pore size 0.65 µm). The filter was rinsed with an additional 4 ml of stop solution and suspended in scintillation fluid (Scintiverse BD, Fisher, Pittsburgh, PA) for assay of radioactivity.

In countertransport experiments, 20 mmol l\(^{-1}\) test elicitor was added to the suspension

![Fig. 1. Time course of lysine uptake. Samples of vesicles suspended in 20 mmol l\(^{-1}\) Ches–Tris and 100 mmol l\(^{-1}\) mannitol at pH 9 were incubated with an equal volume of transport buffer containing 20 mmol l\(^{-1}\) Ches–Tris, 100 mmol l\(^{-1}\) mannitol, 100 mmol l\(^{-1}\) KSCN and 1 mmol l\(^{-1}\) [3H]lysine at the same pH. The results of a single experiment (mean ± s.e.m., N=3) are shown. When not present, error bars are smaller than the symbol.](image)
buffer; the transport buffer was the same as that described above except for the elimination of inhibitor. 5 μl of the valinomycin-treated vesicle suspension was diluted 20-fold into 95 μl of transport buffer. Further manipulation of the mixture followed the steps described above.

The pH of the transport buffer dropped to 9.1 with 20 mmol l⁻¹ dicarboxylic amino acids and to 9.6 with 20 mmol l⁻¹ neutral and dibasic amino acids. However, the initial uptake rates of alanine, leucine, phenylalanine and lysine are pH-independent between pH9 and 10 (Hennigan et al. 1993a, b). Osmotic balance was maintained at the time of mixing in all of our studies.

The initial uptake of alanine, leucine, phenylalanine and lysine without a potassium gradient is linear for at least 9 s, both with and without an inhibitor present; Fig. 2 depicts data obtained with lysine. Symport rates, V, in the presence and in the absence of putative inhibitors were obtained either using linear least-squares fits to the uptake curves over 9 s or by dividing the uptake (corrected for the extrapolated value at $t=0$) after a fixed interval by the length of the interval. Both approaches yielded very similar results. Relative inhibitions are plotted in the figures as $1 - [V(\text{with inhibitor})/V(\text{without inhibitor})]$.

In countertransport experiments, the uptake was measured at a fixed time, $t'$ (see figure legends), and at 1 h when intra- and extravesicular media are at equilibrium (Hennigan et al. 1993b).

Accumulations were calculated using:

$$\text{Accumulation} = \frac{[\text{amino acid}]_{\text{in},t'}}{[\text{amino acid}]_{\text{in, equilibrium}}}.$$

Accumulations significantly greater than 1 indicate that the elicitor and labeled amino acid use a common symporter. This implies that the elicitor would competitively cis-
inhibit the uptake of substrate in an inhibition experiment. This conclusion is necessarily qualitative (in part because a small amount of elicitor is present on the cis-side as well).

Definitive proof of competitive inhibition would require measurement of the appropriate binding and inhibition constants.

Valinomycin and the unlabeled amino acids were obtained either from Sigma Chemical Company, St Louis, MO, or from Aldrich Chemical Company, Milwaukee WI; the radiolabeled amino acids were obtained either from ICN Biopharmaceuticals, Culver City, CA, or from Amersham Corporation, Arlington Heights, IL. Mannitol and KSCN were obtained from Fisher Scientific, Pittsburgh, PA. Unless otherwise identified, L-amino acids were used as substrates and test analogs.

### Results

**Stereospecificity of symport**

The effect of the D-stereoisomer on the uptake of its (natural) enantiomer reveals the symmetry of binding interactions between symporter and substrate. Fig. 3A shows that D-stereoisomers inhibit the uptake of their labeled L-enantiomers in the order, D-lysine > D-leucine > D-phenylalanine > D-alanine. The corresponding elicitation
sequence is D-leucine ≫ D-alanine ≈ D-lysine > D-phenylalanine. D-Phenylalanine, most notably, did not elicit above-equilibrium accumulations of its enantiomer (Fig. 3B). Unlabeled L-amino acids follow the sequence L-leucine > L-lysine ≈ L-alanine > L-phenylalanine in their ability to elicit accumulations of their respective labeled congeners (Fig. 3B).

Fig. 3. (A) Relative inhibition of the uptake of L-amino acids by their enantiomers. A sample of vesicles suspended in 50 mmol l$^{-1}$ AMPD, 100 mmol l$^{-1}$ mannitol and 100 mmol l$^{-1}$ KSCN at pH 10 was incubated with an equal volume of transport buffer containing 50 mmol l$^{-1}$ AMPD, 100 mmol l$^{-1}$ mannitol, 100 mmol l$^{-1}$ KSCN and 1 mmol l$^{-1}$ L-[3 H]amino acid at the same pH. (B) Ratios of accumulations of L-amino acids at 1 min to that at 60 min elicited by D-stereoisomers (dotted columns) and L-stereoisomers (hatched columns). The suspension buffer in the countertransport experiments contained 20 mmol l$^{-1}$ elicitor in addition to mannitol, AMPD and KSCN, whereas the transport buffer was unchanged. Countertransport experiments were initiated by mixing 5 μl of vesicle suspension with 95 μl of transport buffer. The vesicles were preincubated with valinomycin at a concentration of 8 μg mg$^{-1}$ protein. Values are means ± S.E., N = 3.
Sidechain structure

Isomers of leucine that differ in the position of the methyl group (isoleucine and norleucine) were found not to inhibit leucine symport equally (Fig. 4A), although the accumulations of leucine elicited were the same (Fig. 4B). The influence of the aromatic ring, as another example of an uncharged substituent, on phenylalanine symport was examined with 2-aminobutanoic acid and norvaline (which differ in length by a single methylene group) as test inhibitors/elicitors. Both analogs suppressed phenylalanine uptake rates by about 50%. The two analogs were active in the countertransport of phenylalanine, eliciting relative accumulations of approximately 2.2 at 1 min, the time at which Hennigan et al. (1993a) have discovered a broad maximum in the uptake time course, similar to the maximum elicited by phenylalanine itself.

Sidechain length

Neutral amino acids

The initial rate of alanine uptake is inhibited to the same extent by alanine homologs

![Graph A](image1)

![Graph B](image2)

Fig. 4. (A) Relative inhibition of labeled leucine uptake at 6 s by isomers of leucine (from left to right: isoleucine, leucine and norleucine) differing in methyl branch position. (B) Ratios of accumulation of labeled leucine at 1 min to that at 60 min elicited by leucine isomers. See legend to Fig. 3 for experimental conditions. Values are means ± s.e.m., N=3.
with sidechains between 0 (glycine) and 3 (norleucine) methyl groups in length (Fig. 5A). Countertransport measurements (Fig. 5B) show that all of these analogs also elicit the uptake of labeled alanine.

**Basic amino acids**

Four homologous dibasic amino acids [2,3-diaminopropionic acid (DAPA), 2,4-diaminobutanoic acid (DABA), ornithine and lysine] ranging in sidechain length from one to four methyl groups were tested for their ability to inhibit lysine uptake. The inhibiting ability of the test amino acid was found to increase with the length of the sidechain (Fig. 6A). The same amino acids that *cis*-inhibit, efficiently elicit the accumulation of lysine (Fig. 6B), the efficacy increases with increasing chain length, especially for chain lengths in excess of two carbon atoms. To isolate the effect of the distal amino group, norleucine, which is identical to lysine except for the replacement of the terminal amino group by a methyl group, was tested for its effect on lysine uptake. This amino acid suppressed the rate of labeled lysine uptake by approximately 50% and

![Graph A](image.png)

**Fig. 5.** (A) Inhibition of labeled alanine uptake at 6 s by test amino acids with the sidechain lengths shown. (B) The ratio of labeled alanine accumulation at 1 min to that at 60 min as a function of the sidechain length of the elicitor. See legend to Fig. 3 for experimental conditions. Values are means ± s.e.m., N=3.
elicited a 2.5-fold accumulation over equilibrium levels. Arginine proved not to elicit above-equilibrium accumulations of lysine uptake, although it inhibited lysine uptake by about 50%.

**Amino group position**

Aminobutanoic acid isomers that differ in the position of the amino group (2- or α-aminobutanoic acid, 3- or β-aminobutanoic acid and 4- or γ-aminobutanoic acid) affect the initial uptake rate of alanine to different degrees (Fig. 7A). Racemic mixtures of 2- and 3-aminobutanoic acid were used since L-3-aminobutanoic acid was unavailable; 4-aminobutanoic acid has no asymmetric carbon atoms. The countertransport ability of these isomers (Fig. 7B) shows that the 2-amino isomer elicits appreciable alanine uptake, whereas the 3- and 4-amino isomers do not elicit above-equilibrium uptake. β-Alanine was also unable to elicit above-equilibrium accumulation of alanine but inhibited uptake of the α-isomer by approximately 70%. These results show that only α-amino acids are substrates for the zwitterionic amino acid symport pathways.

![Graph A](image1)

![Graph B](image2)

**Fig. 6.** (A) Relative inhibition of labeled lysine uptake (at 6s) by diaminoalkanoic acids of indicated sidechain lengths. (B) Ratios of accumulation of labeled lysine at 40s to that at 60min elicited by lysine homologs with the indicated sidechain lengths. See legend to Fig. 3 for experimental conditions. Values are means ± S.E.M., N=3.
At pH 10, leucine, alanine, phenylalanine and lysine enter *M. sexta* BBMVs predominantly via a broad-spectrum ‘neutral’ amino acid symporter (Hennigan et al. 1993a, b). Determining the effects of substrate chirality on symport may be expected to help elucidate the symmetry of binding interactions between this symporter and its substrates, but strikingly little information is available. In a pioneering study, Hanozet et al. (1984) found that D-alanine shares the pathway of its enantiomer in *Philosamia cynthia* at pH 7.4, despite the presence of a separate symporter. Our data on the relationship between amino acid chirality and symport in *M. sexta* suggest that the effect of chirality is structure-dependent and somewhat more subtle than in *P. cynthia*. Thus, D-leucine and D-lysine inhibited symport of leucine and lysine more than D-alanine and D-phenylalanine inhibited the symport of their enantiomers (Fig. 3A), suggesting that the binding site is less sensitive to the chirality of the longer amino acids, leucine and lysine.

**Discussion**

At pH 10, leucine, alanine, phenylalanine and lysine enter *M. sexta* BBMVs predominantly via a broad-spectrum ‘neutral’ amino acid symporter (Hennigan et al. 1993a, b). Determining the effects of substrate chirality on symport may be expected to help elucidate the symmetry of binding interactions between this symporter and its substrates, but strikingly little information is available. In a pioneering study, Hanozet et al. (1984) found that D-alanine shares the pathway of its enantiomer in *Philosamia cynthia* at pH 7.4, despite the presence of a separate symporter. Our data on the relationship between amino acid chirality and symport in *M. sexta* suggest that the effect of chirality is structure-dependent and somewhat more subtle than in *P. cynthia*. Thus, D-leucine and D-lysine inhibited symport of leucine and lysine more than D-alanine and D-phenylalanine inhibited the symport of their enantiomers (Fig. 3A), suggesting that the binding site is less sensitive to the chirality of the longer amino acids, leucine and lysine.
Not surprisingly, both D- and L-stereoisomers function equally well in eliciting L-leucine symport (Fig. 3B). D-Alanine, D-lysine and D-phenylalanine are increasingly less effective than their enantiomers at eliciting labeled L-amino acid symport. D-Phenylalanine, in particular, does not elicit above-equilibrium accumulations of L-phenylalanine. Considering the inhibition and stimulation data together, we infer (a) that D-phenylalanine and L-phenylalanine are not translocated through the same symporter, perhaps because the inflexible ring accentuates the non-equivalence of the stereoisomers, (b) that the D-alanine and L-alanine symporters may overlap to some extent, since D-alanine trans-stimulates the uptake of L-alanine although it cis-inhibits its uptake only weakly, and (c) that the zwitterionic symporter is insensitive to the chirality of leucine and lysine, perhaps because the sidechains of these amino acids are flexible enough to obscure chiral non-equivalence.

The neutral amino acids taken up by the broad-spectrum symporter are structurally diverse and are similar only to the extent that they have similar pK values. One would therefore expect the symporter to be insensitive to minor structural perturbations of the substrate. This expectation is borne out by the present study. Thus, norleucine and isoleucine are very similar in their ability to inhibit or trans-stimulate the cation-dependent uptake of leucine (Fig. 4). The symporter in M. sexta BBMV, in contrast to the symporter in Ehrlich cells (Oxender and Christensen, 1963), is also relatively insensitive to the sidechain length of alanine homologs (Fig. 5). As mentioned above, aliphatic amino acids of similar length are also acceptable surrogates for phenylalanine. Thus, there is no evidence for specific chemical or physical interactions involving the aromatic ring. Decreasing the sidechain length (alanine, glycine), however, leads to a progressive reduction in the ability to countertransport phenylalanine (Hennigan et al. 1993a, b). The reduction in the ability of shorter homologs to elicit uptake of phenylalanine, and to some extent alanine (Fig. 5), suggests that these compounds could have degenerate transition states, all of which may not be symportable.

Homologs of alanine, leucine and phenylalanine with (a) similar charge distribution and (b) longer sidechains than that of the parent compound seem to function as surrogate symport substrates. Preston et al. (1974) arrived at broadly similar conclusions in their measurement of the affinities of neutral amino acid analogs for the zwitterionic amino acid symporter in rabbit ileum. They found (a) that the sidechain seems to reside in a hydrophobic pocket, which can accommodate a straight chain of four to five carbon atoms, and (b) that branched isomers (leucine and isoleucine) had affinities that were lower by a factor of three than that of the unbranched isomer norleucine. The straight-chain analogs of alanine were found to inhibit alanine uptake to a greater extent as the chain length increased from glycine to norleucine (Daniels et al. 1969). We surmise that the similarity in behavior of the neutral amino acid symporter in disparate organisms reflects an underlying similarity in structure and hints at very closely related origins for this family of carrier proteins.

However, the sidechain length of dibasic amino acids (which are are 70–80% zwitterionic at pH 10) correlates well with their ability to inhibit the uptake of lysine (Fig. 6), suggesting that the distal amino group is a significant marker for the binding site. It is possible that this group, which may be substantially uncharged in the transition state...
owing to charge transfer to the $\alpha$-amino group (Christensen, 1979), participates in hydrogen bonding with the binding site surface. The presence of such orientation-specific substrate–symporter interactions could help to explain the inability of DAPA (which has an aminomethyl sidechain in contrast to the aminobutyl moiety of lysine) to inhibit/elicit lysine uptake, although their complete absence (cf. norleucine, with an apolar $n$-butyl sidechain) may not preclude function as a lysine analog. More generally, such interactions between binding site and putative substrate may enable discrimination among the members of a homologous series. Taken together, the data on alanine homologs and lysine homologs indicate that varying the sidechain length affects symport only if it modifies the charge distribution on the substrate.

In contrast to these examples in which perturbation of substrate structure had only modest effects on symport, displacement of the amino group from the 2-position was found to result in radical decreases in the ability of the compound to cis-inhibit or trans-stimulate the symport of L-alanine (Fig. 7). Since the amino group in the aminobutanoic acid series is at least 50% charged at the pH used in this study, it seems that $-\text{NH}_3^+$ can readily function as a discriminant for alanine symport. The inability to symport $\beta$-alanine highlights the importance of the 2-position for the amino group. Similar restriction to the $\alpha$-isomer has been reported with mammalian neutral amino acid transporters. Daniels et al. (1969) found that the uptake of methionine in rat small intestine was inhibited an order of magnitude more by the 2-isomer of aminobutanoic acid than by the 3- and 4-isomers; however, the 3- and 4-isomers were more effective in inhibiting sarcosine symport. $\alpha$- and $\beta$-alanine displayed analogous behavior. Preston et al. (1974) found the affinity of 3- and 4-$\text{NH}_2$ amino acids for the neutral amino acid transporter in rabbit ileum to be 20- to 30-fold lower than that of the 2-isomer. Of the structural rearrangements investigated, varying the position of the charged amino group has the largest effect on substrate polarity. The dramatic differences that ensue strongly suggest that polarity (and the attendant molecular interactions) may be vital in discrimination between substrate and non-substrate. The restriction of uptake to $\alpha$-amino acids has special significance, since proteins commonly contain only $\alpha$-amino acids.

The structural tolerance exhibited by the zwitterionic symporter appears to be an adaptation to the conflicting demands of selectivity and throughput. A working hypothesis could be that this symporter recognizes amino acid substrates by their charge distribution, i.e. polarity. By this hypothesis, changes in the length of an apolar sidechain would be tolerated because these do not perturb molecular polarity. Alteration in substrate chirality would be tolerated for the same reason. In contrast, the large differences in polarity among 2-, 3- and 4-amino acids result in marked differences in symportability.

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**References**

Substrate structure and amino acid/K⁺ symport


