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Substrate Specificity of the Intestinal Brush-Border Proline/Sodium (*IMINO*) Transporter

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Summary. L-proline uptake via the intestinal brush-border *IMINO* carrier was tested for inhibition by 41 compounds which included sugars, N-methylated, α-, β-, γ- and ε- amino and imino acids, and heterocyclic analogs of pyrrolidine, piperidine and pyridine. Based on competitive inhibitor constants (apparent K_i's) we find that the *IMINO* carrier binding site interacts with molecules which possess a well-defined set of structural prerequisites. The ideal inhibitor must 1) be a heterocyclic nitrogen ring, 2) have a hydrophobic region, 3) be the L-stereoisomer of 4) an electronegative carbonyl group which is 5) separated by a one-carbon atom spacer from 6) an electropositive tetrahedral imino nitrogen with two H atoms. Finally, 7) the inhibitor conformation determined by dynamic ring puckering must position all these features within a critical domain. The two best inhibitors are L-pipecolate (apparent K_i' 0.2 mM) and L-proline (apparent K_i' 0.3 mM).

Key Words sodium-coupled transport · amino acid transport · proline transport · intestinal transport · membrane transport · transport inhibitors · brush-border vesicles

Introduction

Evidence has accumulated that amino acids are transported across plasma membranes by single cells and epithelia via multiple transport pathways (Christensen, 1975; Silbernagl, 1980; Stevens et al., 1984). These pathways include diffusion, Na-independent, and Na-dependent carriers. There are at least seven different carriers with overlapping specificities, and this has hindered in-depth analysis of the kinetics and specificity of a single carrier. Previous studies have suggested that epithelial brush borders possess a rather selective Na-dependent carrier for L-proline (Mircheff et al., 1982; Stevens et al., 1982*a*). The carrier in rabbit jejunum has been designated the *IMINO* carrier (Stevens & Wright, 1983, 1984, 1985; Stevens et al., 1984) because it was strongly inhibited by imino acids, but not by glycine nor most other amino acids. In this paper we describe the structural features of competitive inhibitors of L-proline transport via the *IMINO* carrier. We find that a fairly rigid set of structural prerequisites are needed for strong inhibition of L-proline transport. A preliminary account of some of these observations has already been presented (Stevens & Wright, 1984).

Materials and Methods

Brush-border membrane vesicles were prepared from rabbit jejunum by a Ca^{++} precipitation procedure, and were stored in liquid nitrogen as described previously (*see* Stevens et al., 1984). Vesicles were loaded with 325 mM mannitol, 50 mM KCl, 40 μM valinomycin, and 50 mM HEPES/Tris, pH 7.5. Initial rates of L-proline uptakes were measured during 0 to 4 sec (22°C) using a rapid mix/filter method (*see* Stevens et al., 1982*a*) under initial zero *trans*, voltage-clamped (PD = 0) conditions. The final uptake buffer contained L-[^{3}H]proline (0 to 100 mM), 100 mM NaCl or choline Cl, 50 mM L-alanine, unlabeled inhibitors (0 to 100 mM), 40 μM valinomycin, 50 mM KCl and 50 mM HEPES/Tris at pH 7.5. All inhibitor solutions were adjusted to pH 7.5 with Tris or HEPES, and the osmolarity was adjusted to the loading buffer (intravesicular buffer) with mannitol. Reagents and inhibitors were of the highest purity available from Sigma Chemical Co., St. Louis, Mo. and Aldrich Chemical Co., Milwaukee, Wis., except N-methyl-L-alanine which was obtained from Vega Biochemicals, Tucson, Ariz. L-[2,3,4,5-^{3}H]proline was from Amersham, Arlington Heights, Ill.

Results and Discussion

Definition of the IMINO Carrier

Previous vesicle studies (Stevens et al., 1982*a*) suggested that Na-dependent proline transport occurred via more than one pathway. This was con-

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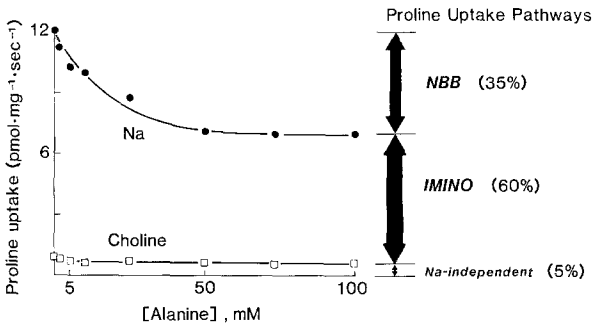

Fig. 1. Proline uptake pathways in small intestine. Labeled proline (50 μM) uptake was measured in triplicate in the presence of *cis* (zero *trans*) unlabeled L-alanine (0 to 50 mM) in 100 mM NaCl or choline chloride. About 60% of total proline uptake occurred via the *IMINO* carrier pathway which is Na-dependent and L-alanine-insensitive; 5% by the combination of Na-independent *L* system plus passive diffusion; and 35% occurred via Na-dependent pathways (primarily the *NBB*, *N*eutral *B*rush *B*order, system). Uptakes are the mean of triplicate determinations. Data points are means and the errors are smaller than the symbols

firmed in the present study by measuring proline uptake as a function of *cis* L-alanine (Fig. 1). We know (Stevens et al., 1982*a*) that L-alanine uptake occurs in rabbit intestinal brush-border membrane vesicles via a Na-independent carrier designated the *L* system, plus a Na-dependent multisubstrate neutral carrier called the *NBB* (*N*eutral *B*rush *B*order) system, plus diffusion. Figure 1 demonstrates the pathways available for proline transport. As the data indicate, 95% of 50 μM proline total uptake is Na-dependent. The Na-dependent fraction asymptotically inhibited by L-alanine includes the *NBB* system responsible for about 35% of total proline uptake. About half of the Na-independent proline uptake (2.5% of the total) was inhibited by alanine via the *L* system, and half (2.5% of the total) constituted nonsaturable diffusion. The remaining 60% of total proline uptake is designated as the *IMINO* carrier of the intestinal brush-border membrane, and is thus operationally defined as the portion of proline uptake which is Na-dependent and L-alanine insensitive.

A converse experiment was carried out (*not shown*) in which 50 μM L-alanine uptake was measured as a function of the *cis* proline concentration. This indicated that proline inhibited about 50% of the Na-dependent L-alanine uptake. Taken together with previous experiments (Stevens et al., 1982*a*) this suggests that the affinity of L-proline for the general neutral carrier is quite low. Peerce and Wright (*unpublished*) find that the K_m for L-proline transport via the alanine-sensitive mode (1.6 mM) is about an order of magnitude higher than that for the *IMINO* carrier (0.23 mM).

Table 1. Noninhibitors of the *IMINO* carrier

Compound[a]
2-pyrrolidone
L-prolinol
Pyrrolidine
Cyclopentanecarboxylic acid
L-glutamic acid
α-AIB[b]
L-alanine
Glycine
β-alanine
GABA[b]
L-lysine
D-glucose
D-mannitol

[a] These compounds gave no inhibition of L-proline uptake via the *IMINO* carrier at [*I*] = 100 mM. For each case the apparent K_i' was >1000 mM or indistinguishable from $K_i' = \infty$. L-alanine here gave no additional inhibition beyond the 50 mM used in the uptake media to define the *IMINO* carrier (*see* text).
[b] Abbreviations: α-AIB, α-aminoisobutyric acid; GABA, γ-amino-*n*-butyric acid.

Inhibition of the IMINO Carrier

In preliminary experiments uptake of L-proline (50 μM) was measured in the presence of 100 mM inhibitors in the uptake buffer. Many gave no inhibition and these are considered to be noninteractive (apparent $K_i' = \infty$) with the *IMINO* carrier (Table 1). This establishes that this carrier is quite distinct from those handling sugars, and neutral, basic and acidic amino acids, and that inhibition by proline analogs is not due to indirect effects; e.g., dissociation of the Na gradient.

Compounds that inhibited L-proline uptake are listed in Table 2, and these were studied in more detail. Apparent inhibitor constants (K_i') were estimated by two methods. One was to measure the inhibition of L-proline uptake (J) at fixed 50 μM proline [Pro] and 10 mM inhibitor [I] concentrations, and estimate the apparent K_i' using the relationship (Preston et al., 1974):

$$\text{apparent } K_i' = (J^I/(J^o - J^I)) \cdot (K_t^* \cdot [I]/([\text{Pro}] + K_t^*)) \quad (1)$$

where proline uptake was measured in the absence (J^o) and presence (J^I) of inhibitor, and K_t^* is the

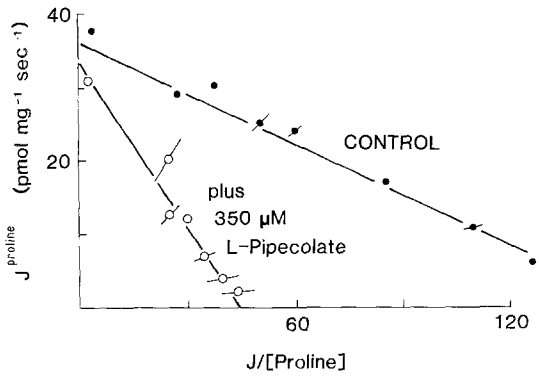

Fig. 2. Competitive inhibition of the *IMINO* carrier by L-pipecolic acid. Proline influx kinetics via the *IMINO* carrier were measured in the presence or absence of 350 μM unlabeled L-pipecolate. Proline influx (J) is plotted as a function of J/[Proline] (abscissa units nl · mg^{-1} · sec^{-1}). The data points are means of triplicate determinations, and the errors, when larger than the points, are shown by the bars. The kinetic parameters were determined by nonlinear regressions. The control J_{max} = 35 ± 2 pmol · mg^{-1} · sec^{-1}, K_i^* = 0.23 ± 0.01 mM. The inhibited J_{max} = 34 ± 5 pmol · mg^{-1} · sec^{-1}, K_i^{app} (slope of inhibited data) = 0.75 ± 0.1 mM, and apparent K_i' = 0.16 ± 0.02 mM

apparent affinity of L-proline uptake in the absence of inhibitor. L-proline transport in the absence of inhibitor occurred by a single saturable system with a J_{max} of 30 to 60 mol · mg^{-1} · sec^{-1} and K_i^* of 230 to 250 μm (*see* Fig. 2; *see also* Stevens & Wright, 1985). Throughout this paper the apparent K_i' serves as a relative index of reactivity with the *IMINO* transporter.

Alternatively, for a smaller number of inhibitors K_i''s were estimated by measuring *IMINO* transport kinetics (L-proline influx as a function of 0.05 to 10 mM [Pro]) in the presence and absence of a fixed concentration of inhibitor [I]. The inhibitor concentrations [I] were either 0.35 mM L-pipecolate, 1.5 mM L-proline-benzyl ester, 2.5 mM N-methyl-L-alanine, or 2.5 mM N-methyl-DL-alanine. The K_i' was obtained from the nonlinear regression analysis of the relationship:

$$J/J_{max} = [Pro]/(K_i^* \cdot (1 + [I]/K_i') + [Pro]). \quad (2)$$

This is illustrated for L-pipecolic acid in Fig. 2, where the Woolf-Augustinsson-Hofstee plot shows that the inhibition was competitive. The apparent K_i' for L-pipecolate was 0.16 ± 0.02 mM using the latter procedure and 0.19 ± 0.03 using the former.

Competitive inhibition was observed for all four compounds tested, and the K_i''s are included in Table 2.

Table 2. Rank of apparent K_i' values for L-proline transport via the *IMINO* carrier[a]

Rank	Inhibitor	Apparent K_i' (mM)	
		Fixed [I], fixed [Pro]	Fixed [I], proline kinetics
1.	L-pipecolate	0.2	0.16
2.	L-proline	0.3	0.23
3.	Proline methyl ester	0.4	—
4.	4-OH-L-proline	0.5	—
5.	Proline benzyl ester	0.7	0.76
6.	Betaine	1.5	—
7.	MeAIB[b]	2.1	—
8.	3,4-dehydroproline	2.2	—
9.	N-methyl-L-alanine	3.1	3.0
10.	N-methyl-DL-alanine	3.2	3.0
11.	BCH[b]	6.9	—
12.	D-pipecolate	7.7	—
13.	Sarcosine	8.7	—
14.	D-proline	10	—
15.	L-azetidine-2-carboxylate	11	—
16.	Phenylalanine	16	—
17.	Thiazolidine-4-carboxylate	19	—
18.	L-proline-*t*-butyl ester	22	—
19.	Prolinamide	28	—
20.	Kainic acid	34	—
21.	Nipecotate	40	—
22.	Picolinate	59	—
23.	Betonicine	70	—
24.	Quinolinic acid	70	—
25.	Pyrrole-2-carboxylate[c]	152	—
26.	Isonipecotate[c]	153	—
27.	Isonicotinate[c]	250	—
28.	Pyroglutamate[c]	350	—
29.	Niacin[c]	375	—

[a] The apparent K_i''s were confirmed using at least two batches of membranes ($n > 3$ for each batch); in the first column the mean of all determinations is shown (± SE < 15%, *not shown*). In the second column competitive inhibition kinetics were also determined for the indicated inhibitors.

[b] Abbreviations: MeAIB, N-(methylamino)isobutyric acid; BCH, 2-amino-2-norbornanecarboxylic acid hemihydrate endo/exo mixture.

[c] Measurable inhibition only at 100 mM (isonicotinic acid at 25 mM).

Nonspecific esterase activity of brush borders was taken to be negligible based on other intestinal vesicle studies (Mircheff & Wright, 1976).

Structure of L-Proline

The three-dimensional structure of L-proline provides a useful starting point in understanding the selectivity of the *IMINO* carrier. Proline is a dy-

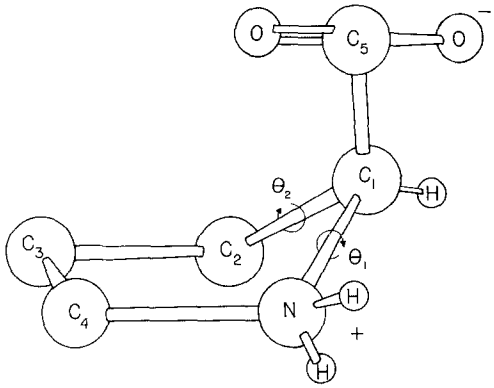

Fig. 3. Three-dimensional structure of zwitterionic L-proline. The structure is based on ^{13}C-NMR data and theoretical considerations (*see* text for refs). Aqueous proline can dynamically interconvert between two half-chair C_2-type conformers due to pseudorotation (puckering) of the ring. Shown is one favored half-chair; the other conformer is obtained by bond twisting through torsion angles θ_1 and θ_2, each about $\pm 40°$. The torsion angles determine the relative intramolecular positioning of *i*) the negatively charged carboxylate, *ii*) the positively charged imino nitrogen with two H atoms, and (*iii*) the hydrophobic ring carbons. The complementary *IMINO* carrier binding site accommodates the L-stereoisomer of one or both half-chairs

namic molecule interconverting between two antisymmetric half-chair conformations. Figure 3 shows one of the principal half-chairs. The structure in aqueous solution is based on theoretical considerations of bond lengths and torsion angles obtained using ^{13}C-NMR and proton resonance spectroscopy (Ramachandran et al., 1970; London, 1978; Abillon, 1982; Govil & Hosu, 1982). The role of conformation stability will be discussed later.

We will now compare and contrast the structure and K_i''s of inhibitors with the structure and affinity of L-proline. The indicated structures are the ionized forms at the pH of these experiments (pH 7.5).

Ring Size

All analogs with $K_i' < 1$ mM are cyclic compounds. The 6-membered ring L-pipecolate (K_i' 0.2 mM) has a greater affinity for the *IMINO* carrier than either the 5-membered L-proline (K_i' 0.3 mM) or 4-membered L-azetidine-2-carboxylate (K_i' 11 mM). It should be noted also that the L-stereoisomer is preferred over the D-stereoisomer; the K_i' values for the ringed stereoisomers (proline and pipecolate) were about 40-fold higher than the K_i' values for the L-stereoisomer (Table 2). However, for the aliphatic N-methyl-alanine, the D- and L- forms gave the same K_i' of 3 mM.

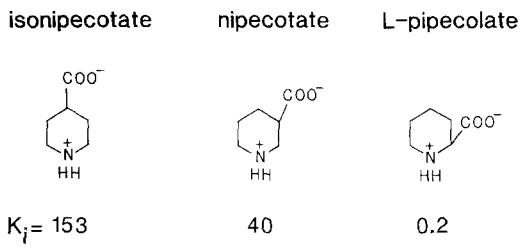

Fig. 4. Effect of the carboxylate position around piperidine rings. The analogous aromatic series—isonipecotate, niacin, picolinate—shows a similar effect. In this and subsequent figures the apparent K_i' values (mM) are obtained from Tables 1 and 2

Relative Position of the Imino and Carboxylate Groups

The importance of the spacing between the imino nitrogen and the carboxylate group is best illustrated by the isomers of piperidine carboxylates (Fig. 4). The K_i' decreases 200-fold on moving the carboxylate group from position 2 to 3 (pipecolate K_i' 0.2 mM *vs.* nipecotate K_i' 40 mM) and another fourfold from position 3 to 4 (isonipecotate K_i' 153 mM). A similar trend is observed with the aromatic pyridinecarboxylates where the K_i' for the 2-pyridinecarboxylate (picolinate, K_i' 59 mM) is substantially lower than the 3- and 4-pyridinecarboxylates (niacin or nicotinic acid, K_i' 375 mM, and isonicotinate, K_i' 250 mM).

Carbonyl Oxygen of the Carboxylate Group

Figure 5 and Table 2 illustrate that replacing the $-\overset{\overset{O}{\|}}{C}-O^-$ group of proline with —H, CH_2OH or =O eliminates interaction of the substrate with the *IMINO* carrier (K_i''s ∞). Nonetheless, fair interaction remains when the $-\overset{\overset{O}{\|}}{C}-O^-$ group is replaced by $-\overset{\overset{O}{\|}}{C}NH_2$ (prolinamide, K_i' 28 mM), $-\overset{\overset{O}{\|}}{C}-O-C_6H_5$ (proline benzyl ester, K_i' 0.7 mM), or $-\overset{\overset{O}{\|}}{C}-O-CH_3$ (proline methyl ester, K_i' 0.4 mM). The relatively high K_i' for proline-*t*-butyl ester (K_i' 22 mM) may be due in part to greater steric hindrance than with the other esters. These data imply that it is the carbonyl oxygen that probably interacts with a positively charged site on the proline carrier. The carboxylate group is separated from the ring nitrogen by one carbon atom.

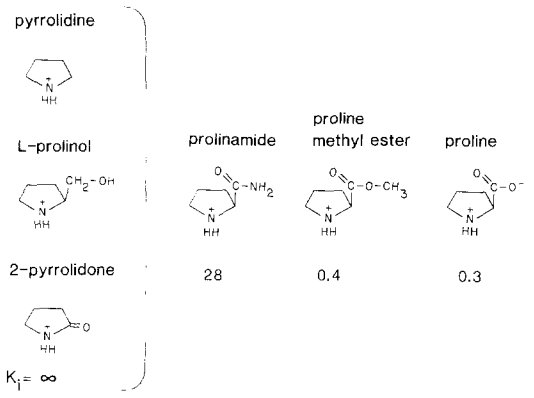

Fig. 5. Requirement for a carbonyl group separated by one carbon atom from the imino nitrogen

Positively Charged Imino Nitrogen with Two H Atoms

A positively charged tetrahedral imino (not amino) nitrogen with two H atoms is essential for substrate interactions with the carrier. The absence of a ring nitrogen in cyclopentanecarboxylate gave no inhibition (Table 1), even with an attached COO^- group. Figure 6 and Tables 1 and 2 demonstrate that only imino acids are effective; the $—NH_3^+$ group of α-, β-, γ- or ε-amino acids (K_i' ∞) prevents interaction with the *IMINO* carrier. This effect is demonstrated by the comparisons in Fig. 7, whereby at pH 7.5 pyrrole-2-carboxylate (K_i' 152 mM) exists in a resonance state which reduces its relative degree of hydrogenation/protonation, betonicine (K_i' 70 mM) is positively charged but is not protonated, picolinate (K_i' 59) is only singly protonated, and proline (K_i' 0.3) has the required $R—NH_2^+—R$ group. The piperidinecarboxylates (e.g., pipecolate, K_i' 0.2) possess two H atoms on the positively charged nitrogen and display low K_i' values, while the pyridinecarboxylates (e.g., picolinate, K_i' 59 mM) possess only one proton on the nitrogen and display relative high K_i' values.

Interactions of Aliphatic Imino Acid

Whereas cyclic imino acids have the greatest affinity for the *IMINO* carrier (Table 2), a number of aliphatic imino acids interact fairly well. In particular, the N-methyl amino acids (sarcosine, N-methyl-L-alanine, MeAIB, and betaine) have K_i' values less than 10 mM (Fig. 8). The imino group or N-methylation dramatically increases the affinity of the respective aliphatics, e.g., pyroglutamate (K_i' 350 mM) *vs.* glutamate (K_i' ∞); N-methyl-L-alanine (K_i' 3 mM) *vs.* L-alanine or α-AIB (K_i' ∞); betaine

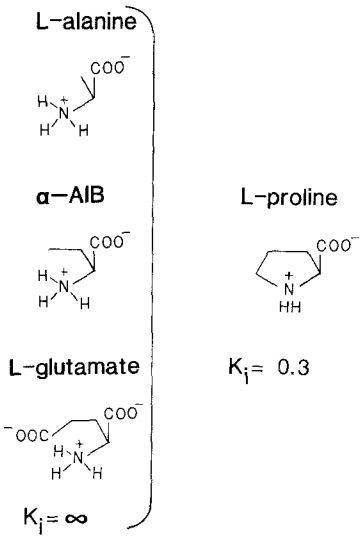

Fig. 6. Effect of the amino ($—NH_3^+$) group of amino acids

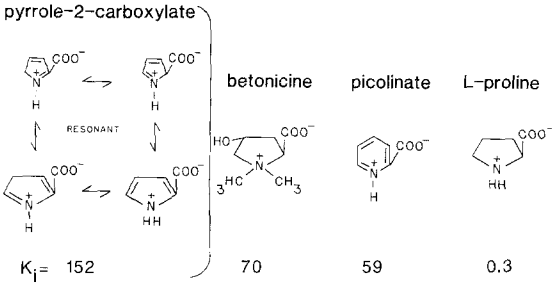

Fig. 7. Effect of two H atoms on the positively charged imino nitrogen. The delocalized electrons of pyrrole-2-carboxylate generate a ring resonance which prevents a fully protonated nitrogen. The betonicine nitrogen is not protonated, but is positively charged

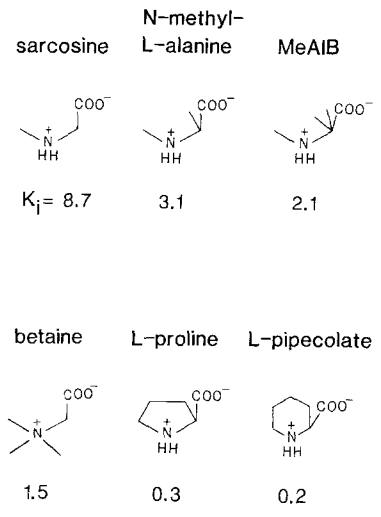

Fig. 8. Aliphatic and cyclic imino acid analog inhibitors

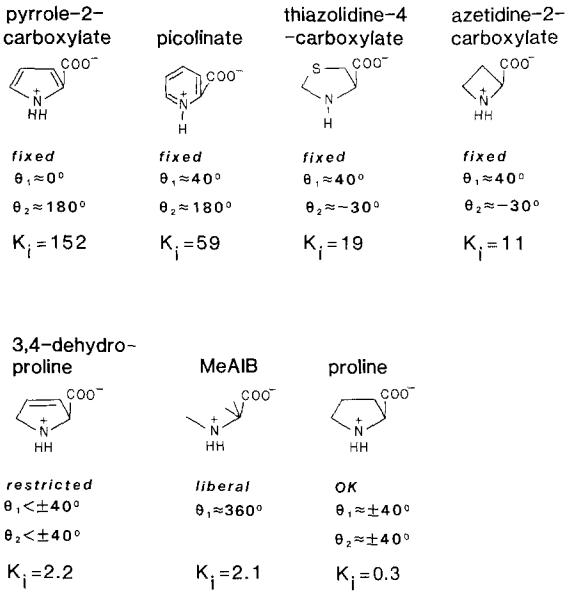

Fig. 9. Estimated bond torsion angles of proline analogs. Pseudorotation energies affect the torsion angles θ_1 and θ_2 of the bonds described in Fig. 3. These must rotate at least ±40° to strategically position the requisite carbonyl, imino and hydrophobic groups for favorable reactivity with the *IMINO* carrier. The torsion angles were estimated using molecular models and computer graphics simulation as outlined in the text

(N,N,N-trimethylglycine, K_i' 1.5 mM) *vs.* sarcosine (N-methylglycine, K_i' 8.7 mM) *vs.* glycine (K_i' ∞). This is not simply a hydrophobic effect, as can be seen by comparing α-AIB (K_i' ∞) *vs.* L-alanine (K_i' ∞) *vs.* N-methyl-L-alanine (K_i' 3.1 mM) (*see* Figs. 5 & 7).

Although two H atoms are optimal for interactions of imino acids with the carrier (*see above*), there is some indication that the properties of the unshared electrons of the imino nitrogen are more important for binding than the proton donors. This is evidenced by the higher affinity for betaine (N,N,N-trimethylglycine, K_i' 1.5 mM) *vs.* sarcosine (N-methylglycine, K_i' 8.7) *vs.* glycine (K_i' ∞), where the inductive effect of the N-methyl groups more than compensates for the loss of the proton donor hydrogen bonds.

Hydrophobic Effects

The greater hydrophobic nature of the N-methyl groups may also play a minor contributing factor, but this is weak as judged by the comparison of betonicine (K_i' 70 mM) with 4-hydroxy-L-proline (K_i' 0.5 mM). A hydrophobic effect is, however, likely to explain in part the decrease in K_i''s of the series (Fig. 8) sarcosine (K_i' 8.7 mM), N-methyl-alanine (K_i' 3 mM), MeAIB (K_i' 2.1 mM), betaine (K_i' 1.5 mM), proline (K_i' 0.3 mM), and pipecolate (K_i' 0.2 mM). This may also explain the interaction of phenylalanine and BCH with the *IMINO* carrier, but it should be noted that the K_i''s of these neutral amino acids are about two orders of magnitude higher than for the optimal imino acids.

The Importance of Conformation and Ring Stability

To summarize thus far, there are six discrete factors which govern a molecule's ability to interact well with the *IMINO* carrier. The molecule should 1) be a heterocyclic ring, 2) have a hydrophobic region, 3) be an L-stereoisomer of 4) an electronegative carbonyl group which is 5) separated by a one-carbon spacer from 6) an electropositive tetrahedral imino nitrogen with two H atoms. An additional integrating factor is 7) the overall molecular conformation which spatially locates each of the other factors.

Not surprisingly, L-proline itself satisfies all the criteria of an excellent inhibitor. However, several of the molecules listed in Table 2 possess these basic requirements and appear to be structurally similar to proline (Fig. 9) but still fail as good inhibitors. The reason is that these analogs cannot assume a critical conformation necessary for binding to the *IMINO* binding site.

The conformation of heterocyclic rings such as proline can be governed by pseudorotation, a puckering wave which undulates around the ring. The puckering of proline places the ring in two preferred half-chair C_2 states (London, 1978). In one preferred conformation shown in Fig. 3 the nitrogen and carbons 2, 3, and 4 are virtually planar, while carbon 1 and the carboxylate group are projected above the plane. The carbonyl group, imino group and hydrophobic regions of the ring are positioned strategically within a domain definable by bond torsion angles θ_1 and θ_2. Certain factors can restrict pseudorotation or reduce intramolecular bond torsions, and thus can affect the conformation. These factors include R group addition, delocalized pi electrons, and sulfur atoms (Riddell, 1980).

In Fig. 9 we estimate the relative puckering restrictions of proline analogs, and we relate the restrictions to the *IMINO* carrier K_i' values. We have roughly estimated the intramolecular torsion angles θ_1 and θ_2 in Fig. 9 in lieu of preseting pseudorotation energies, because energy values are not available for all molecules. However, known pseudorotation energies range from ~0.3 kcal/mole for pyrrolidine (proline) rings to ~3 kcal/mole for sulfur/nitrogen heterocyclic or for aromatic heterocyclic rings (Riddell, 1980). The torsion angles were

estimated using molecular models and limited computer graphics simulation based on X-ray data of the Cambridge Crystallographic Data Base (Allen et al., 1983). The puckering restrictions (Fig. 9) govern the relative positioning of the hydrophobic, imino and carbonyl groups. For the best inhibitors in Table 2 the positioning is within a domain defined by angles θ_1 and θ_2, about $\pm 40°$ for proline (*see* Fig. 3). The low pseudorotational energy barrier of proline (~0.3 kcal/mole) permits movement within this domain. For the poorer inhibitors, the carbonyl group exists in an unfavorable position due to high pseudorotation energy barriers. The restriction of bond torsions can occur in aromatic rings with delocalized pi electrons (pyrrole-2-carboxylate, K_i' 152 mM; picolinate, K_i' 59 mM; 3,4-dehydroproline, K_i' 2.2 mM), and in the strained rings of thiazolidine-4-carboxylate (K_i' 19 mM) and azetidine-2-carboxylate (K_i' 11 mM).

The additional carbon in the 6-membered heterocyclic ring of L-pipecolate permits additional "twisted" ring states (Hendrickson, 1967; Dunitz, 1972) with torsion angles θ_1 and θ_2 extending up to $\pm 60°$ (*cf.* refs. in Allen et al., 1983). This could greatly increase the probability of a preferred conformation which can bind the *IMINO* carrier, and thus may account for the very low K_i' (0.2 mM) of L-pipecolate.

The relatively free rotation of the nitrogen/carbon 1 bond in MeAIB (K_i' 2.1 mM) and in betaine (K_i' 1.5 mM) thus permits these N-methylated aliphatic carboxylates to appear, on a time-average, structurally similar to their more stable puckered cyclic analog, proline (K_i' 0.3).

Combined Effects of the Inhibitor Factors

Many of the test analogs possess a mixture of several inhibitor factors. For example, the delocalized ring electrons of pyrrole-2-carboxylate (K_i' 152 mM) generate at least two factors: 1) the nitrogen is only partially protonated (Fig. 7), and 2) the rigid planar ring restricts critical positioning of the carbonyl group and nitrogen (Fig. 9). These combined effects serve to confirm the single effects demonstrated by the other example analogs.

Imino Transport *vs.* Inhibition

The self-inhibition of L-proline gave a K_i' of 0.23 ± 0.04 mM which compares favorably with the actual transport K_t^* of 0.25 ± 0.01 mM (Stevens & Wright, 1985). Using a voltage-sensitive dye technique we have examined the ability of proline analogs to be themselves transported. We found (Wright et al., 1984) that the ringed structures and betaine were transported, but that the N-methylated aliphatic inhibitors were not themselves transported. The K_t^*'s for the transported analogs were somewhat higher than the K_i''s in Table 2, but the ranking order was the same.

Comparison with Other Studies

Sodium-stimulated proline transport has been demonstrated in a variety of intact tissue (Lerner, 1978) and membrane vesicle preparations including choroid plexus (Ross & Wright, 1984) and kidney (Mircheff et al., 1982). The chicken small intestine has a proline transport component which is inhibited by many aliphatic imino acids, but is unaffected by betaine (Burrill & Lerner, 1972) unlike the present rabbit study (Table 2). Munck (1984) has recently shown that guinea pig intact ileal mucosa possesses a transport system for MeAIB which is strongly inhibited by heterocyclic and N-methylated imino acids. However, unlike the rabbit *IMINO* carrier (Tables 1 and 2), the guinea pig system also interacts with α-amino acids. Studies using membrane vesicles from the guinea pig ileum suggest that Na^+-dependent proline uptake in this species occurs via a carrier not unlike the rabbit jejunal *IMINO* carrier (Hayashi et al., 1980; Stevens et al., 1984).

Sodium-dependent proline uptake has been demonstrated in renal brush-border membrane vesicles (Mircheff et al., 1982). As in rabbit intestine (Stevens & Wright, 1984; Stevens et al., 1984), there are at least two kidney systems for proline, with a high-affinity system contributing 60 to 70% to the total uptake in humans and in rats (McNamara et al., 1976; Ganapathy et al., 1983). The rat kidney high-affinity system (Ganapathy et al., 1983) shares many similarities with the rabbit intestinal *IMINO* system. For example, the rat kidney $K_t^* = 0.23$ mM compared to rabbit intestinal $K_t^* = 0.25$ mM. Also, glycine does not inhibit proline uptake in both tissues. In addition, L-pipecolate itself is transported by both tissues (Wright et al., 1984), and gives low K_i' values against proline transport (0.27 mM in kidney *vs.* 0.18 mM in intestine). Pyroglutamate shows minimal interaction with the high-affinity proline carrier in both tissues (Table 2), although it is transported by a separate carrier with high-affinity K_t^* 20 mM in kidney (Ganapathy et al., 1983). The problem of characterizing substrate specificity for a distinct *IMINO* carrier in kidney may be confounded by the presence of a high-capacity sodium-dependent monocarboxylate carrier which is inhibited by the imino analogs picolinate, niacin, and isonicotinate (Ullrich et al., 1982). However, there is no evidence for the existence of a monocarboxylate carrier in rabbit jejunal brush borders (Stevens et al., 1982*b*; Schell et al., 1983).

Conclusion

The rabbit intestinal *IMINO* carrier is inhibited by competitive analogs which possess only certain well-defined features epitomized by L-proline and L-pipecolate. The substrates with highest affinity are actively puckering heterocyclic rings or N-methylated aliphatic imino acids which place essential R—NH_2^+—R imino, —C(=O)—O^- carbonyl, and hydrophobic regions in the proper L-stereoisomer conformation for reception by the *IMINO* binding site. The features suggest that the binding site contains hydrophobic, positively charged and negatively charged domains. In current (Stevens & Wright, 1985) and future studies of the *IMINO* carrier we will exploit the structural features in experiments designed to describe the kinetic mechanism, and to label and identify the *IMINO* carrier from rabbit intestinal brush borders. Preliminary results with fluorescent group-specific reagents (Wright & Peerce, 1984) indicate that the *IMINO* carrier is composed of a 100,000-dalton polypeptide.

References

Abillon, E. 1982. Geometry of the five-membered ring. Mathematical demonstration of the pseudorotation formulae. *Biophys. Struct. Mech.* **8**:257–270

Allen, F.H., Kennard, O., Taylor, R. 1983. Systematic analysis of structural data as a research technique in organic chemistry. *Acc. Chem. Res.* **16**:146–153

Burrill, P., Lerner, J. 1972. A distinct component of proline transport in chicken small intestine. *Comp. Biochem. Physiol.* **42A**:437–443

Christensen, H.N. 1975. Biological Transport. W.A. Benjamin, Reading, Mass.

Dunitz, J.D. 1972. Approximate relationships between conformational parameters in the 5- and 6-membered rings. *Tetrahedren* **28**:5459–5467

Ganapathy, V., Roesel, R.A., Howard, J.C., Leibach, F.H. 1983. Interaction of proline, 5-oxoproline, and pipecolic acid for renal transport in the rabbit. *J. Biol. Chem.* **258**:2266–2272

Govil, G., Hosu, R.V. 1982. Conformation of Biological Molecules. Springer, Berlin

Hayashi, K., Yamamoto, S., Ohe, K., Miyoshi, A., Kawasaki, T. 1980. Na^+-gradient-dependent transport of L-proline and analysis of its carrier system in brush-border membrane vesicles of the guinea-pig ileum. *Biochim. Biophys. Acta* **601**:654–663

Hendrickson, J.B. 1967. Molecular geometry. VII. Modes of interconversion in the medium rings. *J. Am. Chem. Soc.* **89**:7047–7061

Lerner, J. 1978. A review of amino acid transport processes in animal cells and tissues. University of Main Press, Orono, Maine

London, R.E. 1978. On the interpretation of ^{13}C spin-lattice relaxation resulting from ring puckering in proline. *J. Am. Chem. Soc.* **100**:2678–2685

McNamara, P.D., Ozegovic, B., Pepe, L.M., Segal, S. 1976. Proline and glycine uptake by renal brushborder membrane vesicles. *Proc. Natl. Acad. Sci. USA* **73**:4521–4525

Mircheff, A.K., Kippen, I., Hirayama, B., Wright, E.M. 1982. Delineation of sodium-stimulated amino acid transport pathways in rabbit kidney brush border vesicles. *J. Membrane. Biol.* **64**:113–122

Mircheff, A.K., Wright, E.M. 1976. Analytical isolation of plasma membranes of intestinal epithelial cells: Identification of Na,K-ATPase rich membranes and the distribution of enzyme activities. *J. Membrane Biol.* **28**:309–333

Munck, B.G. 1984. Imino acid transport across the brush-border membrane of the guinea-pig small intestine. *Biochim. Biophys. Acta* **770**:35–39

Preston, R.L., Schaeffer, J.F., Curran, P.F. 1974. Structure-affinity relationships of substrates for the neutral amino acid transport system in rabbit ileum. *J. Gen. Physiol.* **64**:443–467

Ramachandran, G.N., Lakshminarayanan, A.V., Balasubramanian, R., Tegoni, G. 1970. Studies on the conformation of amino acids. XII. Energy calculations on prolyl residue. *Biochim. Biophys. Acta* **221**:165–181

Riddell, F.G. 1980. The conformational analysis of heterocyclic compounds. Academic, New York

Ross, H., Wright, E.M. 1984. Neutral amino acid transport by plasma membrane vesicles of rabbit choroid plexus. *Brain Res.* **295**:155–160

Schell, R.E., Stevens, B.R., Wright, E.M. 1983. Kinetics of sodium-dependent solute transport by rabbit renal and jejunal brush border vesicles using a fluorescent dye. *J. Physiol. (London)* **335**:307–318

Silbernagl, 1980. Renal transport of amino acids and oligopeptides. *In:* Renal Transport of Organic Substances. R. Greger and S. Silbernagl, editors. pp. 93–117. Springer, Berlin

Stevens, B.R., Kaunitz, J.D., Wright, E.M. 1984. Intestinal transport of amino acids and sugars: Advances using membrane vesicles. *Annu. Rev. Physiol.* **46**:417–433

Stevens, B.R., Ross, H.J., Wright, E.M. 1982*a*. Multiple transport pathways for neutral amino acids in rabbit jejunal brush border vesicles. *J. Membrane Biol.* **66**:213–225

Stevens, B.R., Wright, E.M. 1983. The effect of Na^+ on proline transport kinetics in jejunal brush border membrane vesicles. *Fed. Proc.* **42**:1287

Stevens, B.R., Wright, E.M. 1984*a*. Substrate structural requirements for the intestinal *Imino* transport carrier. *Fed. Proc.* **43**:1086

Stevens, B.R., Wright, E.M. 1985. Kinetic model of the brush border proline/sodium (imino) cotransporter. *Ann. N.Y. Acad. Sci. (in press)*

Stevens, B.R., Wright, S.H., Hirayama, B., Gunther, R.D., Ross, H., Harms, V., Nord, E., Kippen, I., Wright, E.M. 1982*b*. Organic and inorganic solute transport in renal and intestinal membrane vesicles preserved in liquid nitrogen. *Membr. Biochem.* **4**:271–282

Ullrich, K.J., Rumrigh, G., Kloss, S., Fasold, H. 1982. Absorption of monocarboxylic acids in the proximal tubule of the rat kidney. II. Specificity for aromatic compounds. *Pfluegers Arch.* **395**:227–231

Wright, E.M., Peerce, B. 1984. Identification and conformational changes of the intestinal proline carrier. *J. Biol. Chem.* **259**:14993–14996

Wright, E.M., Schell, R.E., Stevens, B.R. 1984. Specificity of intestinal brush border proline transport: Cyanine dye studies. *Biochim. Biophys. Acta (in press)*

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