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Transport of L-proline, L-proline-containing peptides and related drugs at mammalian epithelial cell membranes

Review Article

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Summary. Membrane transport of L-proline has received considerable attention in basic and pharmaceutical research recently. Of the most recently cloned members of the solute carrier family, two are ''proline transporters''. The amino acid transporter PAT1, expressed in intestine, kidney, brain and other organs, mediates the uptake of proline and derivatives in a pH gradient-dependent manner. The Na^+-dependent proline transporter SIT1, cloned in 2005, exhibits the properties of the long-sought classical IMINO system. Proline-containing peptides are of interest for several reasons. Many biologically important peptide sequences contain highly conserved proline residues. Xaa-Pro peptides are very often resistant to enzymatic hydrolysis and display, in contrast to Pro-Xaa peptides, a high affinity to the H^+/peptide cotransporter PEPT1 which is expressed in intestinal, renal, lung and biliary duct epithelial cells. Furthermore, several orally available drugs are recognized by PEPT1 as Xaa-Pro analogues due to their sterical resemblance to small peptides.

Keywords: Membrane transport – Imino acids – Proline derivatives – Peptides – PEPT1 – PEPT2 – PAT1 – SIT1 – Proton gradient

Introduction

The end products of intestinal protein digestion are a mixture of free amino acids and small peptides contacting the physical and enzymatic barrier of the small intestinal epithelium (Fig. 1). The epithelial cells are connected by ''tight junctions'', which restrict passage of these nutrients via the paracellular route. These cells possess specific transport systems mediating uptake of their substrates across the apical membranes. Until the 1970s it was generally believed that all proteins are digested to their constituent amino acids and that only the amino acids are absorbed (for review see Matthews, 1975). This dogma was based on the fast discovery of many different amino acid transporters at the time (for review see Christensen 1984, 1989). In addition, new technologies allowing quantification of amino acids but not peptides in body fluids were introduced in the 1950s. In the sixties and seventies several authors were able to demonstrate the appearance of hydrolysis-resistant dipeptides at the serosal side of the intestinal and also the renal epithelium. The uptake was saturable, i.e. carrier mediated, and occurred against a concentration gradient, i.e. it was an active process. The exploitation of intestinal and renal brush-border membrane vesicle technique has made it possible to resolve the long-standing argument over the energy source for active peptide transport (Ganapathy and Leibach, 1983). Intestinal and renal dipeptide uptake is driven by an inside directed H^+ gradient rather than by a Na^+ gradient which is required for uptake of glucose, many amino acids and vitamins. Hence, the view that secondary active transport processes in animal cells are strictly Na^+-coupled was challenged for the first time by this discovery.

Today we know that at the intestinal epithelium, amino acids released during protein digestion and amino acids which are free diet constituents such as taurine are absorbed into enterocytes by at least nine group-specific amino acid transport systems (Ganapathy et al., 1994, 2001; Palacin et al., 1998; Steffansen et al., 2004) (Fig. 1). In the renal nephron, such transporters are responsible for the reabsorption of amino acids which originate primarily from peptide hydrolysis at the renal epithelium and from the primary filtrate. Di- and tripeptides are

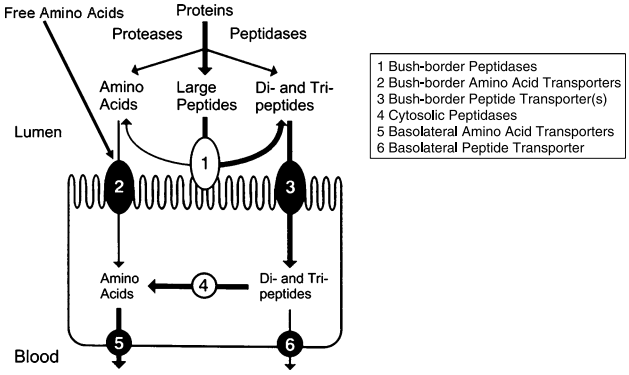


Fig. 1. Protein digestion and absorption of amino acids and peptides at the intestinal epithelium

transported by the H^+/peptide cotransporters PEPT1 (peptide transporter 1), which is present both in intestine and kidney, and by PEPT2 (peptide transporter 2) which is expressed in kidneys but not the intestine. Cytosolic peptidases rapidly hydrolyze most of the di- and tripeptides entering the cells and generate free amino acids (Ganapathy et al., 1994). Inside the cells, the amino acids are either used for cell metabolism or undergo basolateral efflux (Fig. 1). The basolateral cell membranes possess group-specific amino acid transport systems which are responsible for the exit of amino acids from the cell into the circulation and/or uptake of amino acids from extracellular submucosal fluids into the cells (Palacin et al., 1998; Ganapathy et al., 2001). Peptides which are resistant to cytosolic peptidases may be transported intact across the basolateral membrane of intestinal and renal cells by peptide transport systems that have been characterized so far only on a functional level (Fig. 1, Terada et al., 1999, 2000). For example, there was evidence early on for the appearance of intact peptides containing proline and hydroxyproline in the blood. It is generally assumed, however, that the transport of intact peptides across the intestinal epithelium to the blood contributes very little to the total protein absorptive process (Ganapathy et al., 1994; Daniel, 2004).

Epithelial transport of proline, either as free amino acid or in peptide form, is currently receiving considerable attention in basic and pharmaceutical research. The amino acid carriers most relevant for proline absorption, the Proton-coupled Amino acid Transporter PAT1 (or LYAAT1) and the Sodium/Imino-acid Transporter SIT1 (or $IMINO^B$) have been cloned as recently as 2002 and 2005, respectively (Sagne et al., 2001; Boll et al., 2002; Chen et al., 2003; Takanaga et al., 2005b; Kowalczuk et al., 2005). With regard to peptide transporters, several reports focusing particularly on transport of proline-containing peptides and derivatives by PEPT1 and PEPT2 have been published recently. This interest is stimulated by specific chemical, physiological, pathological and biopharmaceutical aspects of proline. There are reports on a possible neuromodulatory role for L-proline and proline-dipeptide derivatives, e.g. effects on food intake (Ortiz et al., 1989), behavioral effects, (Kow and Pfaff, 1991) and modulation of excitatory neurotransmission (Langen et al., 2005). Many biologically important peptide sequences contain highly conserved proline residues (Vanhoof et al., 1995). Prolyl peptide bonds, generally resistant to the action of proteases and peptidases, are the preferred sites of hydrolysis by dipeptidylpeptidase IV (DP IV; for review see Fleischer, 1995). The resulting Xaa-Pro peptides display a high affinity to PEPT1 and PEPT2. Using Xaa-Pro dipeptides and derivatives it was possible to demonstrate experimentally the absolute conformational specificity of the H^+/peptide cotransporter PEPT1 for the *trans* peptide bond conformation (Brandsch et al., 1998, 1999; Bailey et al., 2005). In larger peptides and proteins, *cis-trans* isomerizations are considered a ''molecular switch''. For example, in the neurotransmitter-gated ion channel 5-hydoxytryptamine type 3 receptor, a single proline provides the switch that interconverts the open and closed states (Lummis et al., 2005). Peptidyl-prolyl *cis-trans* isomerases catalyze the slow *cis-trans* isomerization of proline peptide (Xaa-Pro) bonds, thereby accelerating the rate-limiting steps in protein folding (Fischer et al., 1989).

From a biopharmaceutical point of view, proline transporters are very relevant members of the solute carrier families. It has already been shown that PAT1 is able to transport pharmacologically active proline and GABA derivatives (Boll et al., 2002; Anderson et al., 2004; Metzner et al., 2004). As far as peptide transporters are concerned, several orally available angiotensin converting enzyme (ACE) inhibitors derived from Xaa-Pro peptides are recognized by PEPT1 and PEPT2 because of their sterical resemblance to small peptides (Lin et al., 1999; Moore et al., 2000; Shu et al., 2001).

There is at least one possible pathological implication of proline transport, namely the human disease iminoglycinuria. This disease is characterized by a reduced reabsorption of L-proline, glycine and hydroxyproline in the kidney. It was speculated early on that renal imino acid and glycine membrane transporters are affected in this disease either directly, due to defects of the imino transport system(s) or indirectly, for example by defective regulation of the transport process (Goodman et al., 1967; Law and Sardharwalla, 1978).

Transport of L-proline by the H^+/amino acid cotransporters PAT1 and PAT2

PAT expression and transport mechanism

The proton-coupled amino acid transporters (PAT) constitute the recently identified SLC36 family of mammalian membrane transporters (Hediger, 2004). The first member was cloned as the lysosomal amino acid transporter 1 (LYAAT1) from rat brain (Sagne et al., 2001). Subsequently, mPAT1 (SLC36A1, orthologous to LYAAT1) and mPAT2 (SLC36A2) were identified from mouse intestine and embryonic tissue by sequence similarity to amino acid/auxin permease family members of lower eukaryotes (Boll et al., 2002, 2004). The functional characterization was carried out by flux studies and electrophysiology after expression in *Xenopus laevis* oocytes. With regard to pH dependence and substrate specificity these systems showed striking similarity to a transporter functionally described at renal and intestinal cells ten years earlier (Roigaard-Petersen et al., 1989, 1990; Thwaites et al., 1993a, b; Ranaldi et al., 1994). The mRNA of mouse PAT1 is highly expressed in small intestine, colon, kidney and brain but also in lung, liver and spleen (Boll et al., 2002). The mPAT2-mRNA is mainly found in heart and lung. Significant expression was also observed in kidney, testes, liver and spleen. In mouse brain, mPAT2 is expressed in neurons with a different subcellular localization than the lysosomal mPAT1: mPAT2 is found in the endoplasmic reticulum and recycling endosomes of neuronal cell bodies but not in lysosomes (Rubio-Aliaga et al., 2004).

PAT1 has been demonstrated unequivocally, both microscopically and functionally, in epithelial cell membranes. Chen et al. (2003) have cloned the human PAT1 from the intestinal cell line Caco-2, and have comprehensively described the protein structure, substrate specificity and functional parameters of this system in human retinal pigment epithelium (HRPE-) cells. Most importantly, immunolocalization studies revealed the expression of hPAT1 exclusively at the apical cell membrane of Caco-2 cells. With regard to the expression pattern of PAT1 in human tissues, the hPAT1-mRNA was found in small intestine and with lower expression levels also in brain, colon, liver, lung, placenta and testis (Chen et al., 2003; Boll et al., 2003b; Anderson et al., 2004).

PAT proteins mediate electrogenic uphill transport of their substrates into the cells. The transport is energized by a transmembrane electrochemical H^+ gradient directed from outside to inside (Boll et al., 2002; Chen et al., 2003; Anderson et al., 2004; Metzner et al., 2004; Fig. 2). At the

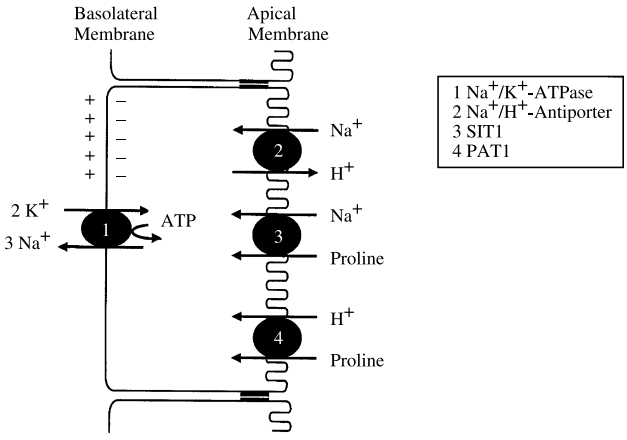


Fig. 2. Proline uptake at a renal or intestinal epithelial cell and its relationship to ion gradients

polarized epithelia of intestine and kidney, this H^+ gradient is known as the ''acidic microclimate''. According to Daniel et al. (1989), at the rat intestinal brush border in vivo, the microclimate pH is about 6.7 in the upper parts of the villi. This H^+ gradient is established and maintained by the activity of the apical Na^+/H^+ antiporter NHE3 (Anderson and Thwaites, 2005). The driving force for the Na^+/H^+ antiporter is the inwardly directed Na^+ gradient established by the Na^+/K^+-ATPase located at the basolateral membrane of polarized epithelial cells (Fig. 2). Since the Na^+/K^+-ATPase is a primary-active carrier and the Na^+/H^+ antiporter a secondary active system, PAT1 is called a tertiary active transport system.

The human PAT1 is the major transport system that mediates the concentrative (uphill) uptake of L-proline into Caco-2 cells. It should be noted that a pioneering study has previously described a H^+-coupled, Na^+-independent L-proline transport in Caco-2 cells (Thwaites et al., 1993b). Many other reports have since been published on the pH gradient-dependent amino acid uptake in Caco-2 cells (Thwaites et al., 1993a, 1995a, 2000). The relevance of these reports was not appreciated until the system was successfully cloned from Caco-2 cells (Chen et al., 2003). In contrast to the current understanding, an earlier report by Nicklin et al. (1992) described the uptake of L-[^{3}H]proline in Caco-2 cells as being Na^+-dependent. These authors were however the first to report the stimulation of L-proline uptake at Caco-2 cells by an outside acidic pH typical for PAT1. The controversy over the modulation of L-proline transport by pH and Na^+ continued with Thwaites et al. (1993b) suggesting a H^+/proline cotransport in Caco-2 cells while Berger et al. (2000) failed to show an effect of an acidic pH on L-[^{3}H]proline flux into the same cell type. These authors however

demonstrated a strong Na^+ dependence for transport. In our studies (Metzner et al., 2004), we did not observe the Na^+ dependence of L-[^{3}H]proline uptake described by Nicklin et al. (1992) or Berger et al. (2000), but confirmed the results obtained by Thwaites and coworkers (1993b). Uptake of both L-[^{3}H]proline and [^{3}H]glycine in Caco-2 cells was Na^+-independent but strongly H^+-dependent. The saturable L-proline uptake was mediated by a single transport system (hPAT1) with an affinity constant (K_t) of 2.0 ± 0.2 mM. We found no functional evidence for a second proline uptake system in these cells (Metzner et al., 2004). The issue was re-evaluated in great detail by Chen et al. (2003) and Anderson et al. (2004). In these studies it was shown very convincingly that only hPAT1 is responsible for L-proline uptake in Caco-2 cells. Comparison of the data with numerous previous reports on epithelial L-proline transport led the authors to conclude that the "H^+/amino acid transporter 1 is the imino acid carrier" (Anderson et al., 2004) referring to the classic Na^+-dependent imino acid transporter functionally identified in the 1960s. The question still remains as to why studies by Nicklin et al. (1992) and by Berger et al. (2000) found a Na^+ dependence for proline uptake in Caco-2 cells whilst others could only identify the pH gradient as the driving force. The reasons for these differences may be reflected in the different culture and/or uptake conditions rather than to differences in Caco-2 subclones used by the different groups. Anderson et al. (Anderson et al., 2004; Anderson and Thwaites, 2005) provided strong evidence for a cooperative functional relationship between H^+-coupled amino acid transport via PAT1 and the Na^+/H^+-exchanger-3. Since the H^+ gradient ultimately relies on the inside directed Na^+ gradient (Fig. 2), experimental sodium depletion will eventually lead to breakdown of the H^+ gradient (Anderson and Thwaites, 2005) and thus alter transport. These authors demonstrated that over short uptake time periods, the transport of ß-alanine (another PAT1 substrate) was not significantly dependent on the presence of sodium in the uptake buffer (Anderson et al., 2004). After 90 min, however, the Na^+-dependent component represented 75% of total ß-alanine uptake. This led the authors to conclude that there may be an indirect Na^+ dependency for transporter activity and this may explain the apparent Na^+ dependence of the imino acid carrier in studies in mammalian intestinal cells. This model by Anderson et al. (2004) was recently challenged by Takanaga et al. (2005b) by cloning the mammalian Na^+-dependent proline transporter SIT1 which is strongly expressed in rat intestine (see below). The cloning of this protein raises another question which might be relevant to the controversy discussed above. Both PAT1 and SIT1 may be expressed by intestinal and renal epithelial cells from several species. However, it is not known whether or under what conditions SIT1 might be expressed in addition to PAT1 in the apical membrane of Caco-2 cells. In our studies we found no functional evidence for the expression of SIT1 (Metzner et al., 2004) but detected mRNA expression for both hSIT1 and hPAT1 in these cells (unpublished results).

Another potential explanation for the conflicting results on the Na^+ dependence of proline uptake in Caco-2 cells could be different culture conditions. The different studies reviewed to date appeared to have cultured Caco-2 cells in different ways with respect to cell supports, seeding density and culture time (Nicklin et al., 1992; Thwaites et al., 1993b; Berger et al., 2000; Metzner et al., 2004; Anderson et al., 2004) and it is not known whether the monolayers used in uptake or flux experiments were confluent in all cases. In experiments where sub-confluent cells are used, the lack of a tight junction between the cells may expose the basolateral membranes to radiolabeled proline. At these membranes, Na^+-dependent amino acid transporters will recognize proline as a substrate (see below).

Substrate specificity of PAT1 and PAT2

The primary substrates of PAT1 are amino acids such as glycine, L-proline and L-alanine (for review see Boll et al., 2004). PAT1 seems to prefer small, unbranched, neutral amino and imino acids. Other substrates are γ-aminobutyric acid (GABA), 3-amino-1-propanesulfonic acid, D-serine, ß-alanine and taurine (Thwaites et al., 1993a, b, 2000; Boll et al., 2002; Chen et al., 2003; Metzner et al., 2004; Table 1). Structures of representative PAT1 substrates are shown in Fig. 3. The system is not stereoselective: PAT1 was shown to translocate D-amino acids such as D-serine, D-proline and D-cycloserine with affinity constants similar or lower than those of the L-isomers (Ranaldi et al., 1994; Thwaites et al., 1995a; Boll et al., 2002; Chen et al., 2003). Boll et al. (2003a) showed that the critical recognition criteria of mPAT1 substrates are the backbone charge distance and the side chain size, whereas substitutions at the amino group are well tolerated. Recent data by our group support the concept that a primary or secondary amino group of either small aliphatic or heterocyclic amino acids is essential for high affinity (Metzner et al., 2004, 2006, this issue). The carrier accepts the 4- and 6-membered rings of proline derivatives as substrates as long as the compound is not decarboxylated (piperidine). Examples with comparatively high

Table 1. Comparison of the amino acid transporters PAT1 and SIT1

Protein name	PAT1	SIT1
Alias	LYAAT1	IMINO[B]
SLC family (Human gene name)	SLC36A1	SLC6A20
Human gene locus	5q33.1	3p21.6
Mechanism	H^+-dependent Na^+-independent	Na^+-dependent Cl^--stimulated H^+-independent
Substrates	Gly, L-Pro, D-Pro, GABA, L-Ala, Taurine, LACA	L-Pro, Pipecolic acid, Sarcosine, LACA (but not GABA, Taurine, L-Ala, D-Pro, Gly)
K_t values for L-proline uptake	2.8 ± 0.1 mM (mouse PAT1 in *X. laevis* oocytes[a]) 2.0 ± 0.2 mM (Caco-2 cells[c])	0.17 ± 0.05 mM (rat SIT1 in *X. laevis* oocytes[b]) 0.25 ± 0.12 mM (OK cells[d])
K_i of LACA	1.8 ± 0.1 mM (Caco-2 cells[c])	0.55 ± 0.15 mM (OK cells[e])

[a] Boll et al. (2002)
[b] Takanaga et al. (2005b)
[c] Metzner et al. (2004)
[d] Ristic et al. (2006)
[e] own unpublished data

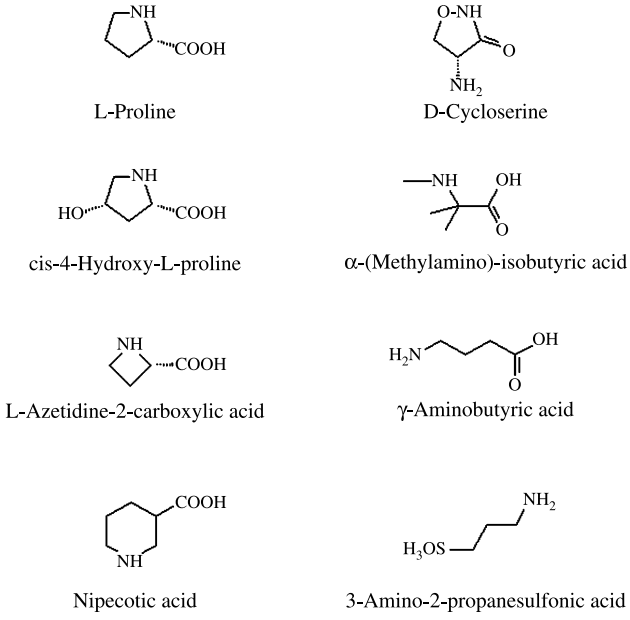


Fig. 3. Structural formulae of representative hPAT1 substrates

affinity are L-azetidine-2-carboxylic acid (LACA) and L- or D-pipecolic acid (Metzner et al., 2004). The carboxy group seems to be important for a high affinity substrate-carrier interaction but it can be replaced by a sulfonyl group. The sulfur-containing amino acid thiaproline represents a hPAT1 substrate with comparably high affinity. Removing the carboxy group as in thiazolidine diminishes the affinity (Metzner et al., 2004).

It should be noted that measurement of L-[^{3}H]proline or [^{3}H]glycine uptake into cells in the presence of unlabeled test compounds allows only the estimation of their potency to inhibit reference substrate uptake. A significant inhibition by a given compound does not allow the conclusion that the effective compound is indeed translocated by the system across the cell membrane. On the other hand, by recording electrophysiological signals it is not possible to find substrates that are transported in an electroneutral mode nor is it possible to identify inhibitors. Such an electroneutral transport mode of both mPAT1 and mPAT2 has been demonstrated for short-chain fatty acids such as acetate, propionate and butyrate (Foltz et al., 2004a). Effective hPAT1-inhibitors have been found recently among amino acids and biogenic amines (Metzner et al., 2005): L-Tryptophan, tryptamine and serotonin are recognized by hPAT1 with affinities similar or even higher than those of the prototype substrates. L-Proline on the other hand did not inhibit L-[^{3}H]tryptophan influx. This result allowed the conclusion that L-tryptophan is not transported by hPAT1, not even in an electroneutral manner. Investigation of membrane potential effects at Caco-2 cells and inward currents in *Xenopus laevis* oocytes expressing mPAT1 revealed that L-tryptophan, indole-3-propionic acid, 5-hydroxy-L-tryptophan, tryptamine and serotonin are not transported by PAT1. They strongly reduced, however, the glycine-induced inward directed current in oocytes.

The structural requirements for mPAT2 substrates are similar to those reported for mPAT1 (Boll et al., 2002; Foltz et al., 2004b). For high affinity binding, however, mPAT2 requires the amino group to be located in an α-position, tolerates only one methyl function attached to the amino group and is highly selective for the L-enantiomers. In contrast to mPAT1, mPAT2 does not transport taurine or GABA. Kennedy et al. (2005) studied the substrate specificity of rat PAT2 and demonstrated that at an extracellular pH of 5.5 and under Na^+-free conditions,

proline uptake was saturable with a Michaelis-Menten constant (K_m) of 172 μM. This result confirmed that, compared to PAT1, PAT2 is the high-affinity transporter. The major restrictions on transport are the side chain size and the backbone length.

Transport of pharmacologically active proline derivatives by PAT1 and PAT2

A very promising approach for the delivery of drugs across epithelial barriers is the exploitation of physiological transport systems. Accordingly, the substrate specificity of carriers, the design of prodrug substrates, the pharmacogenetics relevant to drug transporters and the elucidation of membrane carrier protein structures gained enormous interest in recent years. Well known examples are the drug and prodrug transport via peptide transporters (Daniel and Adibi, 1993; Ganapathy et al., 1998; Bretschneider et al., 1999; see below), the intestinal transport of cationic drugs by the organic cation transporters (Koepsell et al., 2003; Müller et al., 2005) and the therapeutically relevant activity of efflux systems such as the P-glycoprotein.

It is assumed that PAT1 can be used as a new drug delivery route. First, PAT1, but not PAT2, is expressed at epithelial cell membranes. Second, it has been shown early on that PAT1 accepts pharmacologically active amino acids and derivatives such as D-serine, GABA and the antituberculotic agent D-cycloserine as substrates (Ranaldi et al., 1994; Thwaites et al., 1995a; Boll et al., 2002; Chen et al., 2003). We have recently reported that hPAT1 transports numerous therapeutically relevant L-proline derivatives such as 3,4-dehydro-D,L-proline, cis-4-hydroxy-L-proline, LACA and other structures (Metzner et al., 2004; Fig. 3). Such compounds prevent procollagen from folding into a stable triple-helical conformation thereby reducing excessive deposition of collagen in fibrotic processes and the growth of tumors (Takeuchi and Prockop, 1969; Uitto et al., 1984; Ciardiello et al., 1988). Kennedy et al. (2005) has reported that mPAT2 is able to transport LACA and cycloserine.

It would be interesting to investigate whether cis and trans-4-fluoro-L-proline represent hPAT1 substrates. In this regard, it has been reported that cis-4-[^{18}F]fluoro-L-proline, which is used for PET scanning, may be transported in F98 rat glioma cells in a Na^+-dependent manner by the amino acid transport system A (Langen et al., 2002). Very recently the authors demonstrated the stereoselective transport of D-proline and the D-isomer of cis-4-[^{18}F]fluoro-proline at the blood–brain barrier (Langen et al., 2005). As another example, Zhao et al. synthesized new GABA uptake inhibitors that were derived from proline and pyrrolidine-2-acetic acid (Zhao et al., 2005). These compounds should be tested with respects to their oral availability and their interaction with hPAT1.

Transport of L-proline by the Na^+/imino acid cotransporter SIT1

SIT1 expression and transport mechanism

An issue, which was frequently discussed in the past was the driving force of L-proline uptake at the renal and intestinal epithelium (Munck, 1966, 1984; Morikawa and Tada, 1967; Hammerman and Sacktor, 1977; Hayashi et al., 1980; Stevens et al., 1982; Stevens and Wright, 1985; Davies et al., 1987; Vilella et al., 1989; Munck and Munck, 1992; Munck et al., 1994; Urdaneta et al., 1998; Nicklin et al., 1992; Thwaites et al., 1993b; Ingrosso et al., 2000). A key question in the debate is whether proline transport is energized by a proton or by a sodium gradient. As discussed above, the H^+-dependent system PAT1 is considered a major route for proline uptake. Interestingly, the phenomenon that proline uptake into animal cells can be driven by a H^+ gradient was already known from studies in protozoa including the parasite *Leishmania donovani* (Zilberstein and Dwyer, 1985). All along, however, it was clear that this system cannot be identical to the classical IMINO system that had been extensively described on a functional level. There is some confusion in the literature, which is partially due to species specific variants of proline transport: proline uptake at the rat intestine differs distinctly from proline transport in rabbit intestine (Anderson et al., 2004). Moreover, the term *imino transporter* is being used in different context. Therefore, Anderson et al. (2004) emphatically suggested that ''the rat imino acid carrier should be renamed rPAT1 to help reduce further confusion with the IMINO carrier, which is clearly a different entity''.

This classical IMINO carrier is defined as the Na^+-dependent proline transport activity that is resistant to inhibition by alanine (Stevens et al., 1982; Stevens and Wright, 1985; Takanaga et al., 2005b). Just one year ago this system was cloned almost simultaneously by two groups. Takanaga et al. (2005b) cloned and characterized rat SLC6A20 and introduced the acronym SIT1; Kowalczuk et al. (2005) cloned mouse SLC6A20 from kidney and designated the protein $IMINO^B$.

SIT1/$IMINO^B$ is a member of the Na^+- and Cl^--dependent neurotransmitter transporter family SLC6. Proline transport in *Xenopus laevis* oocytes expressing

rSIT1 was Na^+-dependent, Cl^--stimulated and voltage-dependent (Takanaga et al., 2005b) (Fig. 2). Li^+, but not H^+, could substitute for Na^+. rSIT1-mRNA is found in epithelial cells of the whole intestinal tract, in kidney tubule S 3 segments, choroid plexus, microglia and meninges of the brain and in the ovary (Takanaga et al., 2005b). Kowalczuk et al. (2005) showed expression of mouse $IMINO^B$ in brain, kidney, small intestine, thymus, spleen and lung. Romeo et al. (2006) recently studied the tissue distribution of mouse SLC6 amino acid transporters in great detail. Quantitative real-time RT-PCR showed that the mRNA of mSIT1 is abundant in kidney and elevated in small intestine. Importantly, using different experimental approaches, the authors show that mSIT1 localizes to the brush border membrane of proximal kidney tubule and the intestinal epithelium.

Substrate specificity of SIT1

When expressed in *Xenopus laevis* oocytes, rat SIT1 mediated the uptake of L-proline, pipecolate and N-methylated amino acids such as MeAIB and sarcosine (Takanaga et al., 2005b). The affinity constant ($K_{0.5}$) of proline uptake was 0.2 mM. Hence, this carrier displays an approximately ten-fold higher affinity for L-proline than PAT1 (Table 1). The N-methylated derivatives evoked large currents in rSIT1-expressing oocytes but they interacted with rSIT1 with much lower apparent affinity than L-proline. Hydroxy-L-proline was also a strong inhibitor of proline uptake but evoked only a small current which may suggest that this derivative is a non-transported high-affinity inhibitor (Takanaga et al., 2005b). rSIT1-mediated proline transport was insensitive to inhibition by alanine or lysine. This is further evidence for the identity of this system with the classical IMINO carrier. rSIT1 prefers L-proline over its D-stereoisomer (Takanaga et al., 2005b) and is, in this regard, more selective than PAT1. Kowalczuk et al. (2005) reported a very similar substrate specificity and stereoselectivity of this carrier cloned from mouse kidney. The $K_{0.5}$ for L-proline was 0.13 mM. Pipecolic acid had the highest affinity. Amino acids with secondary, tertiary or quaternary amine groups were the preferred substrates of this transporter. Phenylalanine, leucine, alanine and the amino acid analogue aminobutyric acid induced smaller, but significant, currents in *Xenopus* oocytes expressing mouse $IMINO^B$ (Kowalczuk et al., 2005). Ristic et al. (2006) report that in opossum kidney (OK) cells, SIT1 is not only an apical imino acid transporter but also a Na^+-dependent neutral L-amino acid transporter, e.g. for isoleucine and phenylalanine. This agrees with results obtained by Takanaga et al. (2005b) and Kowalczuk et al. (2005) who found that these amino acids induce small but significant currents in *Xenopus laevis* oocytes expressing mouse $IMINO^B$ or rSIT1 or were modest inhibitors of proline uptake, respectively.

Compared to PAT1, the Na^+-dependent proline transporter displays rather limited substrate specificity. Alanine, GABA, D-proline and several other PAT1 substrates are not transported by SIT1. Substrates shared by PAT1 and SIT1 are L-proline, pipecolic acid, sarcosine, MeAIB, hydroxyl-L-proline and LACA. With regard to affinity, SIT1 is the high-affinity transporter and PAT1 the low-affinity transporter. Table 1 summarizes several relevant parameters of SIT1 in comparison to PAT1.

As already stated above, one of the most interesting yet unanswered (but suggested in Fig. 2) questions is that of a possible simultaneous expression of PAT1 and SIT1 in human intestine; hPAT1 as the low-affinity transporter driven by a H^+ gradient and SIT1 as the high-affinity transporter driven by a Na^+ gradient. It is tempting to speculate that both transporters might be expressed at the apical membrane of enterocytes but in different regions of the gastrointestinal tract. In a very recent study, Miyauchi et al. (2005) were able to discriminate between PAT1 and system IMINO in the rabbit kindey: The authors not only cloned and functionally characterized the rabbit PAT1 after heterologous expression in mammalian cells; they detected a transport system with identical characteristics (rabbit PAT1) in renal brush-border membrane vesicles. Most importantly, in the presence of Na^+ and 10 mM L-alanine, i.e. under conditions in which PAT1 transport function is suppressed, a second proline uptake system was detected. This system exhibited the functional characteristics of the IMINO system. Thus, the authors have demonstrated functional activity of both rabbit PAT1 and rabbit SIT1 in the same tissue preparation (Miyauchi et al., 2005).

Since the human $SIT1/IMINO^B$ also functions as a Na^+-dependent proline transporter and is most likely expressed in human kidney, the human SLC6A20 is presently under discussion as a candidate for further investigation of iminoaciduria in man. Takanaga et al. (2005b) found that mSIT1 was dramatically up-regulated in the kidneys of 3-day-old mice, accounting for the known maturation of proline reabsorption in the mouse. In other studies, hPAT1 is believed to be the system which is defective in patients with iminoglycinuria (Miyauchi et al., 2005). Bröer and coworkers (2006a) have already established a complex model involving hPAT1, $IMINO^B$ and the neutral amino acid transporter B^0AT1 to explain

the phenotype of this disease. Identification of the molecular defect underlying iminoglycinuria should be one of the priorities of further efforts in this area.

Other mammalian membrane transporters for L-proline

L-Proline is also a substrate for system ATA2 (amino acid transporter A2, SLC38A2), a subtype of amino acid transport system A. The human ATA2, first cloned from the cell line HepG2 (Hatanaka et al., 2000), consists of 506 amino acids and is ubiquitously expressed in human tissues. The transporter is energized by a Na^+ gradient. Competition experiments using radioactive labeled MeAIB revealed that hATA2 is able to transport several neutral amino acids such as alanine, glycine, serine, proline, methionine, asparagine, glutamine, threonine and leucine (Hatanaka et al., 2000). System A is involved in efflux transport of L-proline across the blood brain barrier (Takanaga et al., 2002). Additional evidence for proline transport by system A was obtained in studies on proline derivatives: In F98 rat glioma cells cis-4-[^{18}F]fluoro-L-proline is transported by system A in a Na^+-dependent manner (Langen et al., 2002). Interestingly, fibroblast cell lines most sensitive to cis-4-hydroxy-L-proline are those in which the activity of the A system is specifically increased (Ciardiello et al., 1988).

Pinilla-Tenas et al. (2003) were first to report transport of proline and hydroxyproline by system ASCT1 (SLC1A4). ASCT1 is a member of the glutamate transporter superfamily and is characterized as a Na^+-dependent neutral amino-acid exchanger. The apparent affinity constants are 704 μM for proline uptake and 33 μM for hydroxyproline uptake. ASCT1 is very likely expressed at the basolateral membranes of enterocytes (Ganapathy et al., 2001; Pinilla-Tenas et al., 2003).

Proline is also transported by PROT (SLC6A7), another member of the Na^+- and Cl^--dependent neurotransmitter transporter family (Chen et al., 2004). PROT is highly specific for proline and is exclusively expressed in the brain. Approximately 80% of PROT proteins are associated with intracellular synaptic vesicle pools. The carrier does not seem to be expressed at mammalian epithelia.

Similarly, a former orphan transporter (V7-3, SLC6A15 gene) of the SLC6 family has recently been characterized as human brain specific Na^+-coupled branched-chain amino-acid transporter 1 (SBAT1, Takanaga et al., 2005a). SBAT1 mediates transport of hydrophobic, zwitterionic α-amino and imino acids with a preference for branched-chain amino acids and methionine ($K_{0.5} = 80–160$ μM). L-Proline is transported with an affinity constant of 0.38 ± 0.02 mM. SBAT1 does not transport charged amino acids, ß-amino acids, glycine and GABA. SLC6A15 has been cloned independently by Bröer and coworkers from mouse but termed B^0AT2 (Bröer et al., 2006b). Transport of neutral amino acids via mB^0AT2 is also Na^+-dependent, chloride-independent and electrogenic. Leucine, isoleucine, valine, proline and methionine are recognized with $K_{0.5}$ values in the 40 to 200 μM range, alanine, glutamine and phenylalanine in the millimolar range. RT-PCR experiments showed significant expression of mB^0AT2 only in brain, lung and kidney. Functionally, B^0AT2-like transport activity was detected in synaptosomes and cultured neurons (Bröer et al., 2006b). Transport function and subcellular distribution of this transporter in kidney and lung have yet to be determined. It should be noted that proline is also transported by the amino acid transporter B^0AT1 (SLC6A19) expressed at the apical membrane of intestinal and renal epithelial cells although with low affinity (Böhmer et al., 2005).

In addition to specific transport, simple diffusion of proline across epithelial barriers might also play a significant role. Apical ion gradient-coupled transporters such as PAT1 and SIT1/IMINO^B accumulate their substrates in the cell, i.e. they transport uphill. Compared to their contribution, proline uptake by simple diffusion might be negligible. Simple diffusion of proline along a downhill concentration gradient may however be significant under physiological conditions.

Transport of L-proline-containing peptides by the H^+/peptide cotransporters PEPT1 and PEPT2

Expression, mechanism and specificity of PEPT1 and PEPT2

Early studies on peptide transport were often done by feeding proteins to live animals and analyzing luminal contents, tissues and blood. Perfusion experiments in vivo and in situ, incubation of everted sacs and rings and the Ussing chamber technique have been used for at least 50 years and are being used today. More advanced cell and tissue preparations such as isolated cells, brush-border membrane vesicles, cell lines, heterologously expressed carriers in *Xenopus laevis* oocytes, mammalian cells or *Pichia pastoris* combined with new analytical methods such as voltage clamp made the molecular characterization and localization of the H^+/peptide cotransporters PEPT1 and PEPT2 possible. From the beginning, the

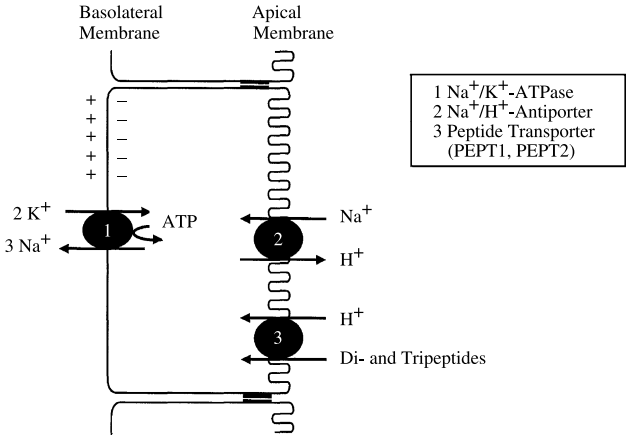


Fig. 4. Peptide transport by PEPT1 or PEPT2 at an intestinal or renal epithelial cell and its relationship to the Na^+/H^+ exchanger and the Na^+/K^+-ATPase

favorite substrates used in intestinal and renal peptide transport studies were proline- and sarcosine-containing dipeptides (Adibi and Soleimanpour, 1974; Addison et al., 1975; Matthews, 1975; Ganapathy et al., 1980, 1981, 1984; Ganapathy and Leibach, 1983; Rajendran et al., 1985). Gly-Sar is highly resistant to both plasma membrane and intracellular dipeptidases (Brandsch et al., 1997) and is available as a radiolabelled tracer with high specific activity.

Using radiolabeled Gly-Pro as a substrate, the group of F. H. Leibach discovered that the renal and intestinal dipeptide uptake is driven by a H^+ gradient and that the transport process is a symport (Ganapathy et al., 1981; Ganapathy and Leibach, 1983). Figure 4 shows the model of dipeptide uptake at a renal or intestinal epithelial cell as established by these authors. The H^+ dependence of intestinal and renal peptide transport has since been confirmed in different tissue preparations and in a variety of animal species such as *C. elegans*, insects, lobster, eel and other fishes, mouse, rat, rabbit and human. In 1994, the rabbit intestinal H^+/di- and tripeptide transporter PEPT1 was finally cloned by the *Xenopus laevis* oocyte expression cloning method (Fei et al., 1994). Shortly thereafter, the renal isoform PEPT2 was cloned and characterized (Liu et al., 1995; Boll et al., 1996). In addition to intestine and kidney, H^+-coupled di- and tripeptide transport across cell membranes by PEPT1 and PEPT2, respectively, has been demonstrated in lung (for review see Groneberg et al., 2004), extrahepatic biliary duct (Knütter et al., 2002), choroid plexus (Teuscher et al., 2000), mammary gland and other tissues (Daniel et al., 1992; Brandsch et al., 1995a, b; Ganapathy et al., 2001; Nielsen et al., 2002; Daniel and Kottra, 2004; Steffansen et al., 2004).

PEPT1 and PEPT2 accept most (but not all, see below) proteinogenic di- and tripeptides and many peptidomimetics as substrates. Free amino acids and larger peptides are not transported. Studies on substrate specificity of peptide transporters performed over the last 40 years have now led to the apparent affinity constants of more than 500 substrates being established. For PEPT1, a wide range of substrate affinity constants with $K_{0.5}$ (K_t, K_m, K_i) values between 2 µM and 30 mM has been determined. The highest apparent affinity reported so far is that of the inhibitor Lys[(Z)NO_2]-Val with a K_i of 2 µM (Knütter et al., 2004). Most dipeptides made of gene-coded amino acids display affinity or inhibition constants in the range of 0.07 to 0.7 mM. In our laboratory, we evaluate K_i values below 0.5 mM as high affinity, K_i values between 0.5 and 5 mM as medium affinity and K_i values higher 5 mM as low affinity. Values above 15 mM we consider with great caution (Brandsch et al., 2004). Using the Caco-2 cell assays we determined a threshold value of 14 mM for cephalosporins and penicillins with respect to their oral availability and this correlates with their affinity to PEPT1 (Bretschneider et al., 1999).

The substrate specificity of PEPT2 is not identical but very similar to that of PEPT1 (for review see Biegel et al., 2006, this volume). For natural di- and tripeptides but not for peptidomimetics, PEPT2 generally displays affinity constants that are about 10fold lower than those at PEPT1. Hence, compared to PEPT1, PEPT2 is called the high-affinity peptide transporter. For PEPT2, we suggested the following classification of substrates and/or inhibitors: (i) K_i values <0.1 mM as high affinity, (ii) K_i between 0.1 and 1 mM as medium affinity and (iii) $K_i > 1$ mM as low affinity. Compounds with apparent affinity constants above 5 mM should not be considered as PEPT2 ligands (Luckner and Brandsch, 2005).

It has to be kept in mind that affinity constants obtained in competition assays using radiolabeled reference substrates give only information about the interaction of these compounds with the carrier protein, not about actual translocation. However, for many representative compounds actual translocation across membranes by transporters has been shown, e.g., by electrophysiological measurements. For a substrate transported by PEPT1 and PEPT2 with high affinity we presently consider the following structural features as essential: (i) L-amino acids, (ii) an acidic or hydrophobic function at the C-terminus, (iii) a weakly basic group in α-position at the N-terminus, (iv) a ketomethylene or acid amide bond, (v) a *trans* conformation of peptide bonds.

This review is limited to proline-containing substrates and derivatives. Recent detailed reviews available in the literature on the topic of peptide transporters are by Ganapathy et al. (1994, 2001), Adibi (1997a, b), Lee (2000), Inui et al. (2000), Nielsen et al. (2002), Brandsch and Brandsch (2003), Brandsch et al. (2004), Daniel and Rubio-Aliaga (2003), Daniel (2004), Daniel and Kottra (2004), Groneberg et al. (2004), Steffansen et al. (2004).

Transport of Xaa-Pro dipeptides by PEPT1 and PEPT2

Proline-containing di- and tripeptides are continuously generated by specific peptidases during proteolysis in the intestinal and renal tubular lumen (Fig. 1). Dipeptidylpeptidase IV (DP IV, EC 3.4.14.5), which is highly active at the renal and intestinal epithelium, releases N-terminal dipeptides of the Xaa-Pro or Xaa-Ala type from larger polypeptides in a sequential manner. These peptides are then taken up into epithelial cells by PEPT1 and PEPT2, respectively (Fig. 1, Brandsch et al., 1995c). Several authors reported the appearance of intact peptides containing proline (both Xaa-Pro and Pro-Xaa) and hydroxyproline in the blood (Prockop et al., 1962; Bronstein et al., 1966).

The interplay between hydrolysis and absorption has been studied in great detail by Morita et al. (1983). During perfusion of rat intestine with the tetrapeptide Leu-Pro-Gly-Gly, the amino acids leucine, proline and glycine were absorbed at a significantly higher rate from the tetrapeptide than from an equivalent amino acid mixture. Within the lumen, Leu-Pro and Gly-Gly were the major hydrolytic products. Using Gly-Pro-ß-naphthylamide, a specific DP IV substrate, the authors were able to show that this enzyme is mainly localized to the brush border membrane and that it is responsible for the hydrolysis of the tetrapeptide into the two dipeptides Leu-Pro and Gly-Gly (Morita et al., 1983). At the kidney epithelium, the relation between hydrolysis and absorption of proline-containing peptides has been investigated using ß-casomorphin-5 (ßCM5: Tyr-Pro-Phe-Pro-Gly) (Miyamoto et al., 1987). As a pentapeptide, ßCM5 is not transported by PEPT1 or PEPT2 nor is it cleaved to any significant extent by peptidases other than the DP IV. Miyamoto et al. (1987) using brush-border membrane vesicles could show that the pentapeptide was hydrolyzed by DP IV to di- and tripeptides which were then transported into the vesicles via the peptide transport system. This mechanism has been confirmed very elegantly using a unique model, the Fischer 344 rats from the Japanese Charles River Inc. In contrast to the Charles River (U.S.A.) Fischer 344 rats, these rats are DP IV deficient (Tiruppathi et al., 1990). Renal brush-border membrane vesicles of DP IV negative rats failed to hydrolyze Tyr-Pro-[^{3}H]Phe-Pro-Gly and did not accumulate radiolabel from the parent peptide (Tiruppathi et al., 1990). Urine analysis revealed that the DPP IV-negative rats excreted proline- and hydroxyproline-containing peptides in significantly increased amounts compared with control rats. These data provided conclusive evidence for the obligatory role of DPP IV in the renal handling of proline (and hydroxyproline)-containing peptides (for review see Brandsch et al., 1995c).

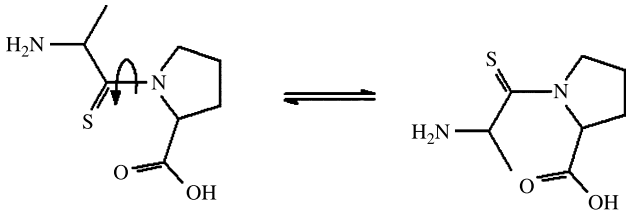
Most Xaa-Pro dipeptides are high-affinity substrates for the H^+/peptide transporters PEPT1 and PEPT2. Constitutively expressed PEPT1 in intestinal Caco-2 cells apparently has K_i values in the range of 0.15 mM (Ala-Pro) to 1.2 mM (Pro-Pro) (Brandsch et al., 1999; Table 2). By comparison, constitutively expressed PEPT2 in renal SKPT-2 cells shows much lower values, having a K_i of 0.02 mM for Ala-Pro and 0.04 mM for Val-Pro.

We were able to experimentally show the conformer specificity of PEPT1 using a novel Xaa-Pro derivative (Brandsch et al., 1998). An Ala-Pro derivative was synthesized by replacing the peptide bond with an isosteric thioxo peptide bond. A striking feature of Ala-ψ[CS-N]-Pro (Fig. 5) was that the compound had a *trans* peptide

Table 2. Examples for inhibition constants of Xaa-Pro and Pro-Xaa dipeptides and derivatives substrates of hPEPT1

Dipeptide/Derivative	K_i (mM)	*Trans* content (%)
H-Ala-Pro-OH	0.15 ± 0.02	64 ± 1
H-Leu-Pro-OH	0.18 ± 0.01	60 ± 1
H-Glu-Pro-OH	0.26 ± 0.01	55 ± 1
H-Gly-Pro-OH	0.30 ± 0.02	52 ± 2
H-Arg-Pro-OH	0.39 ± 0.05	70 ± 2
H-Tyr-Pro-OH	0.53 ± 0.03	30 ± 1
H-Trp-Pro-OH	0.54 ± 0.08	24 ± 1
H-Pro-Pro-OH	1.2 ± 0.1	51 ± 2
H-Sar-Pro-OH	2.5 ± 0.1	62 ± 2
H-Ala-D-Pro-OH	15 ± 2	
H-Ile-pyrrolidide	>20	
H-Ile-thiazolidide	6.3 ± 1.6	
H-Ala-Pip-OH	0.06 ± 0.01	58 ± 3
H-Ala-ψ[CS-N]-Pro-OH	0.30 ± 0.02	62 ± 1
H-Pro-Leu-OH	0.47 ± 0.04	
H-Pro-Tyr-OH	0.73 ± 0.02	
H-Pro-Phe-OH	1.9 ± 0.1	
H-Pro-Arg-OH	2.5 ± 0.1	
H-Pro-Ala-OH	9.5 ± 0.4	
H-Pro-Glu-OH	20 ± 1	
H-Pro-Gly-OH	22 ± 2	

K_i values ± SE derived from inhibition of [^{14}C]Gly-Sar uptake in Caco-2 cells and % *trans* conformers in *cis*/*trans* equilibrium are from Brandsch et al. (1999)



Trans *Cis*

Fig. 5. Structure and *cis*/*trans* isomerization of Ala-ψ[CS-N]-Pro

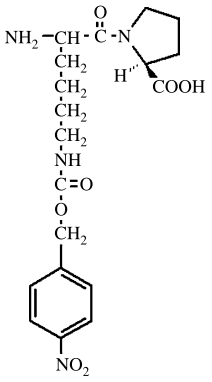
bond content of 96% with only 4% in the *cis* conformation when dissolved in an uptake buffer (pH 6.0). Interconversion of isomers proceeded slowly towards *cis*/*trans* equilibrium (=62% *trans*, 38% *cis*). Although Ala-ψ[CS-N]-Pro was recognized by PEPT1 with high affinity, only the *trans* conformer interacted with this transporter. Moreover, in a second approach, actual *trans* conformer-specific uptake of Ala-ψ[CS-N]-Pro was demonstrated by analyzing the intracellular content of Caco-2 cells and the extracellular medium during incubation using capillary electrophoresis. This study provided first direct evidence that a H^+/peptide cotransport system selectively binds and transports the *trans* conformer of a peptide derivative (Brandsch et al., 1998; Bailey et al., 2005). The advantage of Ala-ψ[CS-N]-Pro for such investigations was its sufficiently low isomerization rate. In contrast, the *cis*/*trans* interconversion of natural Xaa-Pro dipeptides occurs at room temperature within seconds to minutes (Fischer, 1994; Ma et al., 1995). To show whether the phenomenon of conformer specificity applies for natural Xaa-Pro dipeptides as well, and to elucidate additional decisive structural factors relevant for dipeptide-carrier interaction, the affinity of short amide and imide derivatives for the intestinal H^+/peptide symporter was investigated by measuring their ability to inhibit [^{14}C]Gly-Sar transport at PEPT1 (Brandsch et al., 1999). The K_i values of Xaa-Pro dipeptides in *cis*/*trans* equilibrium and those of the corresponding Xaa-Ala dipeptides (all *trans*) were determined. With the exception of Pro-Ala, the affinity constants of Xaa-Ala dipeptides were found to be lower than those of the Xaa-Pro dipeptides. Analyzing the individual peptide bond conformations of Xaa-Pro dipeptides, a striking correlation between the *cis*/*trans* ratios (*trans* contents 24–70%, Table 2) and the affinity constants was observed. After correcting the K_i values for the incompetent *cis* isomers, the $K_{i\ corr}$ values of most Xaa-Pro dipeptides were in a close range of 0.1 to 0.16 mM and very similar to the K_i values of the respective Xaa-Ala dipeptides (range 0.08 to 0.16 mM). It was concluded that PEPT1 accepts *trans* conformers of zwitterionic Xaa-Pro dipeptides regardless of size, hydrophobicity and aromatic nature of the N-terminal amino acid. Lower affinities of Lys-Pro, Arg-Pro and Pro-Pro as well as Lys-Ala, Arg-Ala and Pro-Ala indicate that additional factors such as charge affect their binding at PEPT1. It should be noted that *cis* conformers can be detected not only in aqueous solutions of all Xaa-Pro dipeptides but also for derivatives such as Ala-pipecolic acid, Sar-Pro and Xaa-Sar dipeptides (Table 2, Brandsch et al., 1999). The conformer specificity of PEPT1 for tripeptides has not yet been investigated.

Transport of Pro-Xaa dipeptides by PEPT1

Inoue et al. (2001) developed a highly sensitive and reliable HPLC method for the determination of prolyl dipeptides in serum. According to their measurements, the concentrations of Pro-Hyp, Pro-Gly and Pro-Pro in serum were $0.64 \pm 0.35\,\mu M$, $0.078 \pm 0.047\,\mu M$ and $0.022 \pm 0.016\,\mu M$, respectively. The serum concentration of free proline was $177 \pm 43\,\mu M$ (Inoue et al., 2001). Much higher concentrations are expected at the intestinal and renal brush border during protein hydrolysis. Several authors have reported that dipeptides which have a methylated amino nitrogen (e.g. Sar-Gly and Sar-Pro) or an amino nitrogen as part of the imino ring (e.g. Pro-Gly and Pro-Pro) have much lower apparent affinities than Gly-Pro (Addison et al., 1975; Thwaites et al., 1994; Eddy et al., 1995). We studied this phenomenon in detail in competition assays using Caco-2 cells and [^{14}C]Gly-Sar as a reference substrate (Brandsch et al., 1999, 2004). Pro-Xaa dipeptides displayed K_i values between 0.5 and >20 mM, reflecting much lower and more diverse affinity for PEPT1 than Xaa-Pro dipeptides (Table 2). For example, Pro-Leu (0.5 mM), Pro-Tyr (0.7 mM), Pro-Pro (1.2 mM) and Pro-Phe (2 mM) represent effective inhibitors of [^{14}C]Gly-Sar uptake but fall into the medium affinity category (Brandsch et al., 1999). Pro-Ala, Pro-Asp, Pro-Ser, Pro-Glu and Pro-Gly display very low affinity or no affinity at all. It is concluded that the decisive factors for binding of these dipeptides to PEPT1 are probably the hydrophobicity of the C-terminal amino acid and the rigidity of the structure. The latter conclusion is implied by the result that the affinity of Pro-Pro is 8-fold higher than that of Pro-Ala (Brandsch et al., 1999, 2004).

Development of the first non-transported, high-affinity inhibitors of PEPT1 and PEPT2

The first non-transported high-affinity peptide transporter inhibitor was developed using Lys-Pro (Knütter et al.,



Lys[Z(NO_2)]-Pro

Fig. 6. Structural formula of the H^+/peptide cotransporter inhibitor Lys[Z(NO_2)]-Pro

2001; Theis et al., 2002). Adding the benzyloxycarbonyl (Z)-group to the ε-amino group of the Lys-residue turned Lys-Pro from a normal PEPT1 substrate into a non-transported derivative with significantly higher affinity. Addition of the NO_2-function in *para* position to the hydrophobic ring moiety enhances the affinity for binding of the resulting Lys[Z(NO_2)]-Pro (Fig. 6) to PEPT1 further while maintaining its inability for electrogenic cotransport. Lys[Z(NO_2)]-Pro binds with high affinity to PEPT1 ($K_i = 5–10\,\mu M$), competes with various dipeptides for uptake into cells but is not transported. Synthesis and functional analysis of Lys-Lys derivatives containing benzyloxycarbonyl (Z) or 4-nitrobenzyloxycarbonyl (Z(NO_2)) side chain protection groups provided a set of inhibitors that reversibly inhibited the uptake of dipeptides by PEPT2 with K_i values as low as 10 ± 1 nM (Theis et al., 2002). This is the highest affinity of a PEPT2 ligand ever reported. Based on structure-function relationships it was concluded that (i) the spatial location of the side chain amino protecting group in a dipeptide containing a diaminocarbonic acid and (ii) its intramolecular distance from the α-C-atom (Fig. 6) are key factors for the transformation of a substrate into an inhibitor of PEPT2.

Transport of Xaa-Pro and Pro-Xaa derived drugs by PEPT1 and PEPT2

Besides β-lactam antibiotics, ACE inhibitors such as captopril and enalapril are peptidomimetics that have been intensely studied with regard to their interaction with PEPT1 and PEPT2. Even so, the transport of these compounds is still a matter of much controversy. For instance, the affinity constants for enalapril reported in the literature range from 0.07 mM to >20 mM. Several authors consider the orally-active ACE inhibitors captopril and enalapril as PEPT1 substrates and itemize them as such in their reviews. In a study using Caco-2 cells, transport of captopril and enalapril maleate by PEPT1 was suggested (Thwaites et al., 1995b). In contrast, Moore et al. (2000) found no affinity of captopril, enalapril, enalaprilat and lisinopril for PEPT1 ($K_i > 20$ mM). This is in agreement with results from our laboratory in Caco-2 cells where we failed to show that captopril or enalapril acted either as substrate or inhibitor of PEPT1. In these the affinity constants for these compounds were >30 mM and 12 mM, respectively (Brandsch et al., 2004). Interestingly, Moore et al. (2000) reported a comparably very low K_i value of 110 μM for fosinopril.

In rabbit renal brush border membrane vesicles, an affinity constant of 6 mM was determined for enalapril (Lin et al., 1999). The values for fosinopril and zofenopril were 55 and 81 μM respectively. The authors report that the affinity of ACE inhibitors for PEPT2 was strongly correlated with their lipophilicity. The surprisingly high affinity of fosinopril was confirmed in independent studies using cell lines (Shu et al., 2001). In these studies, transport of fosinopril in Caco-2 cells expressing PEPT1 (Ganapathy et al., 1995) and in SKPT cells expressing PEPT2 (Brandsch et al., 1995a) produced K_i values of 35.5 μM and 29.6 μM respectively (Shu et al., 2001). Intracellular accumulation of fosinopril was 3 to 4 times higher from the apical side compared to uptake from the basolateral side. In further experiments the authors could show unequivocally that fosinopril was transported intact by PEPT2 and PEPT1 by a proton-coupled, saturable process. Quinapril is a noncompetitive, non-transported inhibitor (Shu et al., 2001; Nielsen et al., 2002; Herrera-Ruiz and Knipp, 2003).

A series of proline dipeptide derivatives were studied in perfused rat intestinal segments by Bai et al. (1991). The intestinal permeabilities of phenylpropionylproline, phenylacetylproline, N-benzoylproline, and hippuric acid were significantly reduced by coincubation with dipeptides and cephradine, suggesting that these dipeptide analogues without an α-amino group are transported by the peptide carrier. Ezra et al. (2000) studied the transport of peptidyl-bisphosphonates. Transport of Pro-[^{3}H]Phe-[^{14}C]pamidronate and Pro-[^{3}H]Phe-[^{14}C]alendronate in an in situ single-pass perfusion study was inhibited by Pro-Phe (for which we measured a K_i value of 1.9 mM at Caco-2 cells, Brandsch et al., 1999). The authors postulated that the oral absorption of bisphosphonates can be improved when given as peptidyl prodrugs. Other Pro-Xaa derivatives reported on in the literature are the orally

active hydroxyprolylserine derivatives trans-4-L-hydroxyprolyl-L-serine (JBP923) and cyclo-trans-4-L-hydroxyprolyl-L-serine (JBP485) (Liu et al., 2000). JBP923 is almost completely absorbed from the gastrointestinal lumen. The result that the compound was able to inhibit the H^+-dependent transport of Gly-Sar in brush-border membrane vesicles suggests the involvement of peptide transporters (Liu et al., 2000).

Structural information about substrate-transporter-interaction is highly relevant for a rational design of peptidomimetic drugs, which may be administered orally and/or which shall be reabsorbed very efficiently at the renal epithelium. Recently, the most rigid dipeptide analogue Ala-ψ[CS-N]-Pro was used as template for the identification of pharmacophore features of PEPT1 substrates (Gebauer et al., 2003; Biegel et al., 2005). The relationship between the physico- and stereochemical properties and the affinity for PEPT1 was investigated for 106 dipeptides, tripeptides, peptide derivatives and ß-lactam antibiotics. 3D-QSAR studies using comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analyses (CoMSIA) were performed. By combining five CoMSIA contour maps, i.e. steric, electrostatic, hydrophobic/hydrophilic, hydrogen bond donor and hydrogen bond acceptor, the recognition elements of dipeptides, tripeptides and ß-lactam antibiotics which are favorable for binding to PEPT1 have been identified. These 3D-QSAR models have now allowed us to predict the affinity constants of new compounds (Gebauer et al., 2003; Biegel et al., 2005).

Other mammalian membrane transporters for L-proline-containing peptides

In addition to PEPT1 and PEPT2, the proton oligopeptide cotransporter family (SLC15) also consists of the peptide/histidine transporters, PHT1 and PHT2, that have been characterized, but in much less detail (Herrera-Ruiz and Knipp, 2003; Daniel, 2004; Daniel and Kottra, 2004). In 1997, Yamashita et al. reported the cloning and functional expression of PHT1 from rat brain. When expressed in *Xenopus laevis* oocytes, PHT1 mediated H^+-dependent high-affinity uptake of histidine as well as di- and tripeptides. The system is also expressed in retina and placenta. The protein sequence reveals very weak similarity with PEPT1 and PEPT2 (32% and 27%, respectively). Rat PHT2 encodes a protein of 582 amino acid residues showing 49% identity with rat PHT1 (Sakata et al., 2001). It is expressed mainly in spleen, thymus and lung. Neither PHT1 nor PHT2 have been analyzed systematically with respect to their substrate specificity (Daniel and Kottra, 2004). Very recently, however, the human PHT1 was cloned and functionally characterized in COS-7 cells (Bhardwaj et al., 2006). Surprisingly, uptake of Gly-Sar by this transporter is almost negligible and is unaffected by pH. Several other di- and tripeptides such as Gly-Leu and Gly-Gly-Leu at a concentration of 1 mM were able to inhibit hPHT1-mediated uptake of histidine by less than 50%. The study did not contain kinetic experiments which would make it possible to assess the affinity of the peptides. Since none of these studies included proline-containing peptides, it is not known to the author whether PHT1 and PHT2 transport such compounds under physiological conditions.

As stated above, a peptide transport function has been found at the basolateral membranes of renal and intestinal cells (Terada et al., 1999, 2000; Fig. 1). Uptake of [^{14}C]Gly-Sar across the basolateral membrane into Caco-2 cells cultured on filters was less sensitive to extracellular pH than uptake across the apical membrane by PEPT1. Moreover, the uptake did not proceed against a concentration gradient. These results suggest that the basolateral system is a facilitative peptide transporter whereas PEPT1 is an active transporter (Terada et al., 1999). The same group also studied Gly-Sar uptake across the basolateral membrane of renal MCDK cells cultured on filters (Terada et al., 2000). Major differences in the affinity to dipeptides and a different pH profile suggest that intestinal and renal basolateral peptide transport is mediated by different proteins. Again, it is not known whether these peptide transport systems accept proline-containing peptides as substrates.

Proline is also a conspicuous amino acid in substrates of another peptide transporter referred to as Transporter associated with Antigen Processing (TAP). This ATP-dependent system is not expressed at epithelial cell membranes but at the membrane of the endoplasmatic reticulum. The peptides, which constitute TAP substrates, are generated from endogenous proteins in the proteosomal pathway. Human TAP preferentially recognizes peptides 8–16 residues in length (Uebel et al., 1997). They are translocated by TAP into the ER lumen and assembled with major histocompatibility complex class I molecules. Using combinatorial peptide libraries, Uebel et al. elegantly showed that proline is the most disfavored amino acid in position two of the nonamer peptides tested for recognition by TAP (Uebel et al., 1997). Since the major contribution to binding strength is from the peptide backbone it might be speculated that conformational specificity plays a role for TAP.

Table 3. Tissue localization of cell membrane transporters for L-proline and L-proline-containing peptides

Protein	Gene	Localization
PAT1	SLC36A1	intestine (epithelial cells: apical membrane), brain (neurons: lysosomes), kidney, lung, liver, spleen
PAT2	SLC36A2	lung, kidney, heart, muscle, testes, liver, spleen, thymus, spinal cord, brain (neurons: endoplasmatic reticulum, endosomes[a])
SIT1, IMINO[B]	SLC6A20	intestine, kidney (epithelial cells: apical membrane), brain (choroid plexus, microglia, meninges), ovary
ATA2	SLC38A2	widespread; at epithelial barriers predominantly at abluminal cell membranes
ASCT1	SLC1A4	widespread; at epithelial barriers predominantly at abluminal cell membranes
PROT	SLC6A7	brain (glutamatergic neurons)
B^0AT2, SBAT1	SLC6A15	brain (neurons: synaptosomes), lung, kidney
B^0AT1[b]	SLC6A19	intestine, kidney (epithelial cells: apical membrane)
PEPT1	SLC15A1	intestine, kidney, extrahepatic biliary duct (epithelial cells: apical membrane)
PEPT2	SLC15A2	kidney; lung (bronchial epithelium: apical membrane), glia, choroid plexus (epithelial cells: apical membrane); mammary gland

[a] Rubio-Aliaga et al. (2004)
[b] Low affinity for L-proline (Böhmer et al., 2005); for further details see Hediger (2004) or http://www.bioparadigms.org/slc/menu.asp

Conclusions

At polarized epithelia, proline specific peptidases, proline transporters and peptide transporters contribute in a complex and fine-tuned interplay to membrane proline transport. The mammalian tissues most thoroughly studied in this respect are the epithelial barriers of kidney and intestine. It has been well established that protein digestion products are absorbed into intestinal cells predominantly in the form of di- and tripeptides. Proline-containing di- and tripeptides are generated mainly by dipeptidylpeptidase IV. Probably all Xaa-Pro dipeptides and some Pro-Xaa dipeptides are taken up into the cells by the H^+/peptide cotransporters. The transport of Xaa-Pro dipeptides is conformer specific for the *trans* peptide bond. Free proline is absorbed from the lumen by the H^+/amino acid symporter PAT1 and/or the Na^+/imino acid symporter SIT1.

Inside the cells, most Xaa-Pro and Pro-Xaa dipeptides are hydrolyzed by the dipeptidases prolidase (EC 3.4.13.9) and prolinase (EC 3.4.13.8), respectively, to their constituent amino acids. Administration of excess amounts of proline-rich proteins or peptides will overload degrading enzymes and lead to the appearance of significant amounts of proline-containing peptides in the blood. In all other cases, the individual amino acids are transported out of the cell via basolateral amino acid transporters. Basolateral transport of proline is most likely mediated by systems ATA2 and ASCT1. Although to a different degree, proline and proline-peptide transporters accept derivatives with sterical resemblance to their natural ligands as substrates.

Table 3 summarizes the proline-transporting SLC members known so far on a molecular level. In the near future the remaining amino acid and peptide transporters will be cloned and the substrates for the so-called orphan transporters will be identified.

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