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Defining Minimal Structural Features in Substrates of the H^+/Peptide Cotransporter PEPT2 Using Novel Amino Acid and Dipeptide Derivatives

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ABSTRACT

The peptide transporter PEPT2, expressed in a variety of tissues, including kidney, lung, and the central nervous system, mediates the uphill transport of di- and tripeptides, as well as a variety of peptidomimetic drugs. To identify the essential molecular features of substrates that determine affinity and transport by PEPT2, we synthesized a series of amino acid derivatives as well as modified dipeptides. Kinetic constants for the interaction of test compounds with PEPT2 were obtained in a competition assay using *Pichia pastoris* yeast cells expressing mammalian PEPT2. The two-electrode voltage-clamp technique in *Xenopus laevis* oocytes was used to assess the substrate's electrogenic transport properties. Whereas ϖ-amino fatty acids showed no affinity for PEPT2, the introduction of a single carbonyl group into the backbone increased both affinity and transport currents more than 30-fold. ϖ-Amino fatty acids, at their amino or carboxyl group coupled to an alanine residue, allowed us to determine the importance of the spatial position of functional groups within the molecule. Affinity and transport function declined by elongating the ϖ-amino acid chain when located in the N-terminal position, whereas the elongation in the carboxyl terminal with an N-terminal alanine caused less pronounced effects. The results clearly establish that a free N terminus, a correctly positioned backbone carbonyl group, and a carboxylic group that is in a suitable distance from the intramolecular carbonyl function and the amino terminal head group are the main features for substrate recognition and transport by PEPT2. This information provides the framework for a rational design of peptidomimetic drugs for delivery via PEPT2.

The mammalian H^+/peptide transporter PEPT2 is responsible for the rapid and efficient uptake of a large number of different di- and tripeptides as well as various peptidomimetics across the plasma membrane of kidney tubular cells, lung epithelia, and mammalian brain cells (Ganapathy et al., 1983; Daniel et al., 1991; Liu et al., 1995; Boll et al., 1996; Leibach et al., 1996). The transporter acts as a high-affinity/low-capacity system transporting its substrates in a proton-coupled electrogenic mode (Boll et al., 1996). The intestinal peptide transporter PEPT1, belonging to the same family as PEPT2, mediates the uptake of di- and tripeptides and derivatives into intestinal epithelial cells in a similar mode. PEPT1 has been studied extensively with respect to substrate specificity, and findings allowed the formulation of preliminary predictive models for substrate recognition (Borner et al., 1998; Brandsch et al., 1998, 1999; Döring et al., 1998d; Meredith et al., 1998). In previous studies (Döring et al., 1998d), we showed that PEPT1 transports ϖ-amino fatty acids electrogenically with affinities that are similar to those of native dipeptides. By using ϖ-amino fatty acids of increasing chain length, the minimal molecular requirements for substrate-PEPT1 interactions were defined as free terminal amino and carboxylic groups separated by at least four methylene groups. Another study demonstrated that amino acid arylamides are recognized by PEPT1 as high-affinity substrates (Borner et al., 1998) and that the replacement of the native peptide bond by a thioxo-peptide bond is also well-tolerated by PEPT1 (Brandsch et al., 1998). These and other data obtained in different expression systems for PEPT1 of different species were used for the first modeling approaches to obtain a template for the interaction within the substrate-binding domain (Bailey et al., 2000).

It is well-established that PEPT2 has similar but not identical requirements for substrate recognition and transport. Generally much higher affinities are determined for the ma-

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ABBREVIATIONS: Gly-Gln, glycyl-L-glutamine; PPB, potassium phosphate buffer; MES, 2-(*N*-morpholino)ethanesulfonic acid; 4-ABA, 4-aminobutanoic acid; 5-APA, 5-aminopentanoic acid; Ala, alanine; D-Phe-Ala, D-phenylalanyl-L-alanine; I, current; I-V, current-voltage; $I_{Gly\text{-}Gln}$, current elicited by glycyl-L-glutamine.

jority of PEPT2 substrates. However, systematic studies on the minimal structural features of substrate binding are not yet available for the cloned PEPT2 protein covering a larger set of substrates. Because PEPT2 is expressed in a variety of tissues, including kidney, lung, and the central nervous system, defining its substrate template could also be of importance for a rational design of pharmacologically active compounds for a targeted delivery.

To define the key substrate-recognition criteria, we synthesized a variety of amino acid arylamides as well as modified dipeptides containing ϖ-amino acids as probes. Using *Pichia pastoris* yeast cells expressing PEPT2 (Döring et al., 1998b), we first determined the affinity of the test compounds for interaction with PEPT2 by their ability to compete for the uptake of the radiolabeled dipeptide D-Phe-Ala. To be able to differentiate between compounds that only interact with the substrate-binding site of the transporter and those that are electrogenically transported via PEPT2, we performed an electrophysiological analysis of inward currents induced by the substrates in *Xenopus laevis* oocytes expressing PEPT2 (Boll et al., 1996). Although partially different test compounds were used in the present study compared with those used to study PEPT1, experiments were performed under the same experimental conditions as those reported previously (Döring et al., 1998a). This format also permits a comparison of the observed differences between the two transporters with respect to substrate specificity for binding and transport.

Experimental Procedures

Materials. Female *X. laevis* oocytes were purchased from Nasco (Fort Atkinson, WI). Glycyl-L-glutamine (Gly-Gln) was obtained from Sigma Chemie (Deisenhofen, Germany); D-Phe-Ala was purchased from Bachem (Heidelberg, Germany). Custom-synthesized [^{3}H]D-Phe-Ala (specific radioactivity, 40 Ci/mmol) was obtained from Biotrend (Köln, Germany), and collagenase A was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Substrates and precursors were purchased from Sigma Chemie. All noncommercially available test compounds were synthesized according to standard procedures (Wunsch, 1974; Barth et al., 1980) in peptide chemistry.

***P. pastoris* Strains and Transport Assays in Yeast.** Cultures of *P. pastoris* strains expressing PEPT2 were prepared as described previously (Döring et al., 1998b). Cells were centrifuged at 3000*g* for 10 min and formed into a pellet, washed twice with 100 mM potassium phosphate buffer (PPB; pH 6.5), and resuspended to 5×10^7 cells/20 μl of PPB. Uptake measurements were performed at 22 to 24°C using a rapid-filtration technique on 96-well filter plates (filter material HATF type, 0.45-μM pore size; Millipore Corporation, Eschborn, Germany). In brief, uptake was initiated by mixing 20 μl of the cell suspension with 30 μl of PPB containing 0.05 μCi of [^{3}H]D-Phe-Ala either with or without competitors (final concentration, 0.0001–20 mM). After 15 min of incubation, the uptake was terminated by the addition of 200 μl of ice-cold PPB followed by filtration. The filters were washed four more times with 200 μl of PPB, removed from the plate with a punch, and transferred into vials. Radioactivity associated with the filter was measured by liquid-scintillation counting.

***X. laevis* Oocytes Expressing PEPT2 and Electrophysiology.** Surgically removed oocytes were separated by collagenase treatment and handled as described previously (Boll et al., 1996). Individual oocytes were injected with 30 nl of RNA solution containing 30 ng of rabbit PEPT2 cRNA. All electrophysiological measurements were performed after 3 to 6 days by the incubation of oocytes in a buffer composed of 88 mM NaCl, 1 mM KCl, 0.82 mM $CaCl_2$, 0.41 mM $MgCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, and 10 mM MES/Tris at pH 6.5 (modified Barth's solution).

The two-electrode voltage-clamp technique was applied to characterize responses in current at different transmembrane potentials after substrate addition to oocytes expressing PEPT2. In brief, oocytes were placed in an open chamber with a volume of 0.5 ml and continuously superfused with modified Barth's solution or with solutions of Gly-Gln and/or substrates to be tested. Electrodes with a resistance between 1 and 10 MΩ were connected to a TEC-05 amplifier (NPI Electronic, Tamm, Germany). PEPT2 expressing oocytes were voltage-clamped at −60 mV, and current-voltage (I-V) relationships were measured using short pulses (100 ms) separated by 200-ms pauses in the potential range from −160 to +40 mV. I-V measurements were made immediately before and 30 s after substrate application, when current flow reached steady state. Currents evoked by PEPT2 at a given membrane potential were calculated as the difference of the currents measured in the presence and absence of substrate. Each substrate was tested against the maximal inward current elicited by 5 mM Gly-Gln, allowing for a comparison of current recordings that are independent of the level of functional expression of various oocyte batches. The electrophysiological measurement data are representative of at least two independent experiments and different oocyte batches.

Statistics. All calculations (linear as well as nonlinear regression analyses) were performed using Prism software (GraphPAD, San Diego, CA). At least two independent experiments with three replicates were carried out for each variable. Data are given as mean ± S.E.M.

Results

The apparent substrate affinity of each of the test compounds was determined by using a competition assay taken from PEPT2 expressing *P. pastoris* yeast cells. Additionally, substrates were studied for electrogenic transport via PEPT2 by the two-electrode voltage-clamp technique in PEPT2 expressing *X. laevis* oocytes. This approach, derived from two independent transport assays, allowed us to distinguish between compounds that only interact with the substrate-binding site and those that are also transported across the membrane after binding.

Role of the Carbonyl Group for Substrate Affinity of PEPT2. As recently shown (Döring et al., 1998d), ϖ-amino fatty acids are transported by the intestinal peptide transporter PEPT1 when expressed heterologously in yeast cells and oocytes. Electrogenic transport of ϖ-amino fatty acids requires the two head groups (amino and carboxyl terminals) to be separated by at least four methylene groups serving as an intramolecular spacer. These compounds, therefore, clearly defined the minimal molecular requirements for a substrate interaction with PEPT1. For this reason, we used 5-aminopentanoic acid (5-APA) as the smallest amino fatty acid recognized and transported by PEPT1 to determine whether the same minimal substrate requirements apply to PEPT2 (Table 1). Whereas the affinity of 5-APA for PEPT1 was 1.14 mM (Döring et al., 1998c), which is in the range of native dipeptide substrates of PEPT1, its affinity for PEPT2 was 7.28 ± 1.32 mM (Fig. 1A), which was far below that which is characteristic for PEPT2 substrates (Amasheh et al., 1997). Similar results were obtained for several other ϖ-amino fatty acids (data not shown). In addition, the very low affinity of 8-aminooctanoic acid for interaction with PEPT2 has also been demonstrated in LLC-PK1 cells transfected with rat PEPT2 (Terada et al., 2000). It therefore can

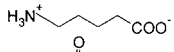
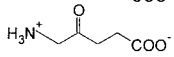
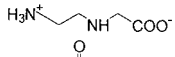
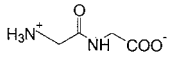
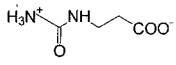
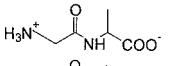
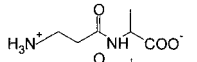
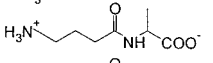
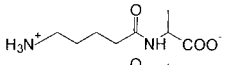
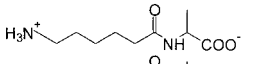
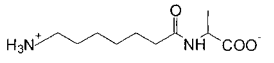
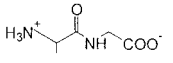
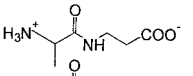
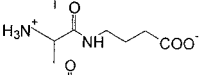
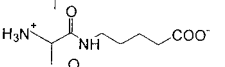
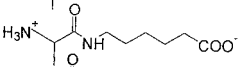
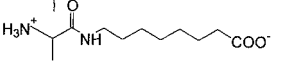
be concluded that ϖ-amino fatty acids are not substrates of PEPT2. However, when we incorporated a carbonyl group into the backbone of 5-APA (yielding 5-amino-4-oxopentanoic acid), the affinity increased more than 30-fold to 0.22 ± 0.01 mM (Fig. 1A, Table 1), which is comparable with that of normal PEPT2 substrates; electrogenic transport characteristics typical of PEPT2 were obtained. It is likely that 5-amino-4-oxopentanoic acid can serve as a substrate because it mimics the dipeptide Gly-Gly, in which the peptide bond is replaced by a ketomethylene function. On the contrary, the Gly-Gly derivative *N*-β-aminoethyl-Gly, with a reduced peptide bond lacking the carbonyl function but still containing the nitrogen of the Gly-Gly peptide bond, shows no interaction with PEPT2 (K_i > 10 mM). Thus, it is obvious that indeed the carbonyl group plays the essential role.

Importance of the Location of the Carbonyl Group within the Backbone. Because ϖ-amino fatty acids are very flexible, it is difficult to obtain information about the spatial location of functionally important groups within such molecules. To determine the importance of the molecular distance between the terminal amino function and the backbone carbonyl group, we used a series of modified dipeptides consisting of an ϖ-amino acid with a different chain length at the N terminus and an alanine residue in the C-terminal position (Table 1). The smallest of these compounds, glycyl-alanine, displayed an affinity of 0.11 ± 0.01 mM, as expected for a standard dipeptide (Fig. 2A). Lengthening this molecule by just one methylene group incorporated between the amino group and the amide bond (β-Ala-Ala) already reduced the affinity 8-fold to 0.93 ± 0.01 mM (Fig. 2A, Table 1). In parallel to the decrease in affinity, the substrate-evoked inward currents decreased to only 50% $I_{Gly\text{-}Gln}$ (Fig. 2B). Successive lengthening of the ϖ-amino acid chain by methylene units resulted in a further dramatic decline in affinity to 5.61 ± 0.22 mM for 4-ABA-Ala and up to 25.1 ± 6.4 mM for 7-aminoheptanoic acid-Ala (Fig. 2A, Table 1). Carbamoyl-β-Ala, a Gly-Gly derivative in which the amide bond is shifted to the N terminus so that a methylene unit between the N-terminal amino group and the amide bond is lacking, showed no interaction with PEPT2. This signifies that a

TABLE 1

Properties of ϖ-amino fatty acids and ϖ-amino acyl-Ala derivatives by interaction with PEPT2

Apparent K_i values ± S.E.M. were calculated from IC_{50} values derived by nonlinear regression analysis of data shown in Figs. 1A, 2A, and 3A. Percentages of $I_{Gly\text{-}Gln}$ were taken from the recordings of the I-V relationships shown in Figs. 1B, 2B, and 3B representing the current evoked by 5 mM concentrations of the tested compound and compared with 5 mM of the dipeptide Gly-Gln at a membrane potential of −100 mV.

Compound	Structure	K_i	$I_{Gly\text{-}Gln}$
		mM	%
5-Aminopentanoic acid		7.28 ± 1.32	34
5-Amino-4-oxopentanoic acid		0.22 ± 0.01	103
N-β-aminoethyl-Gly		>10	0
Gly-Gly		0.16 ± 0.05	117
Carbamoyl-β-Ala		>10	0
Gly-Ala		0.11 ± 0.01	136
β-Ala-Ala		0.93 ± 0.01	50
4-ABA-Ala		5.61 ± 0.22	0
5-APA-Ala		3.60 ± 0.29	12
6-AHA-Ala		6.54 ± 0.22	24
7-AHA-Ala		25.1 ± 6.4	21
Ala-Gly		0.07 ± 0.01	95
Ala-β-Ala		0.39 ± 0.09	26
Ala-4-ABA		0.12 ± 0.03	50
Ala-5-APA		0.06 ± 0.02	91
Ala-6-AHA		0.76 ± 0.07	21
Ala-8-AOA		0.37 ± 0.03	62

minimal distance between the carbonyl function and the N-terminal amino group must be kept to obtain an interaction with the peptide carrier (Fig. 1A, Table 1).

Importance and Sterical Localization of the C-Terminal Carboxylic Group. To obtain more information on the importance of the terminal carboxylic group of substrates on the affinity for PEPT2, we synthesized a series of dipeptides with opposite sequences carrying an N-terminal alanine residue and C-terminal ϖ-amino acids of increasing chain length (Table 1). In the first substrate of this series, alanyl-glycine, the distance from the amide bond to the terminal carboxylic group is prototypical for dipeptides. Consequently, alanyl-glycine shows a high affinity of 0.07 ± 0.01 mM (Fig. 3A, Table 1) and an expected high substrate-evoked inward current of 95% $I_{Gly\text{-}Gln}$ (Fig. 3B). The elongation of the ϖ-amino acid chain by one methylene group (Ala-β-Ala) reduced the affinity by approximately 4-fold to 0.39 ± 0.09 mM, and currents—although measured under substrate saturation of PEPT2—declined to 26% $I_{Gly\text{-}Gln}$. Introducing additional -CH_2 units (Ala-4-ABA and Ala-5-APA), however, increased both the affinity and currents to 0.12 ± 0.03 mM and 50% $I_{Gly\text{-}Gln}$ in the case of Ala-4-ABA and 0.06 ± 0.02 mM and 91% $I_{Gly\text{-}Gln}$ in the case of Ala-5-APA, respectively (Fig. 3, A and B; Table 1). The introduction of additional methylene groups (Ala-6-aminohexanoic acid) again resulted in a 6-fold decline in affinity (0.76 ± 0.07 mM) and lower substrate-evoked inward currents (Fig. 3, A and B; Table 1).

Ala-8-aminooctanoic acid, a compound that, formally seen, exceeds the chain length of a tripeptide, displayed a surprisingly good affinity of 0.37 ± 0.03 mM and caused currents of 62% $I_{Gly\text{-}Gln}$. Peptides possessing more than three amino acids are known by size restriction not to be accepted by the substrate-binding domain of PEPT1 (Fei et al., 1994). However, depending on the chain length of the ϖ-amino acid, our considerably flexible test compounds may more or less adopt

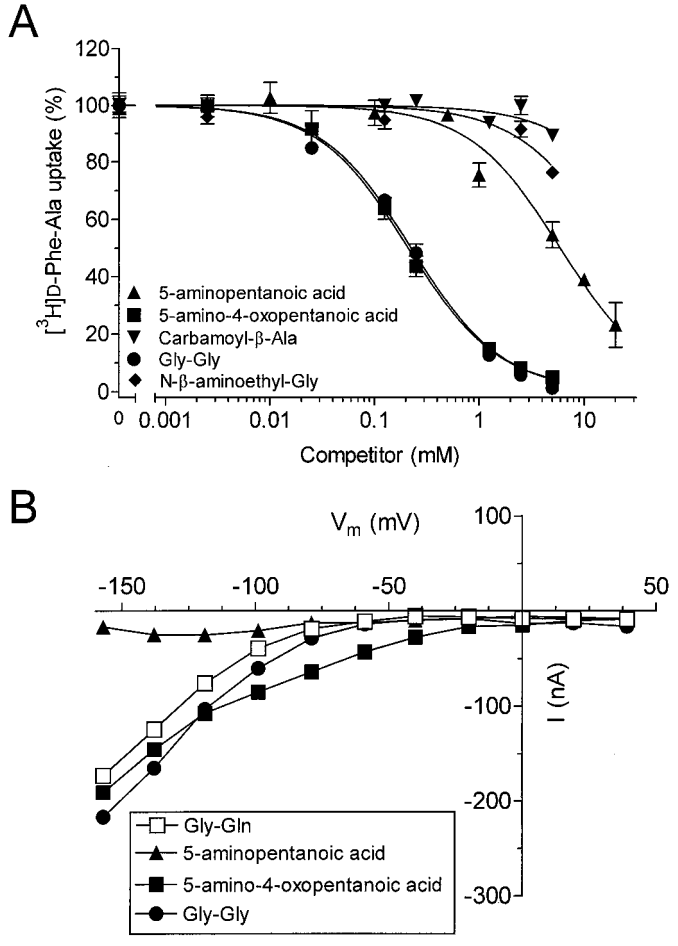


Fig. 1. Characteristics of the interaction of ϖ-amino fatty acids with the H^+/peptide cotransporter PEPT2. A, uptake of [^{3}H]D-Phe-Ala (2 μCi/ml) into *P. pastoris* cells expressing PEPT2 was measured at pH 6.5 in the presence of increasing concentrations (0.001–20 mM) of ϖω-amino fatty acids after a 15-min incubation and was normalized against the optical density (OD) of cells. Uptake measured in the absence of competitors (301 ± 19 pmol/15 min/OD) was taken as 100%. B, steady-state I-V relationships were measured by the two-electrode voltage-clamp technique in oocytes expressing PEPT2 superfused with modified Barth's solution at pH 6.5 and 5 mM Gly-Gln or the corresponding ϖ-amino fatty acids. The membrane potential was stepped symmetrically to the test potentials shown, and substrate-dependent currents were obtained as the difference measured in the absence and the presence of 5 mM substrate.

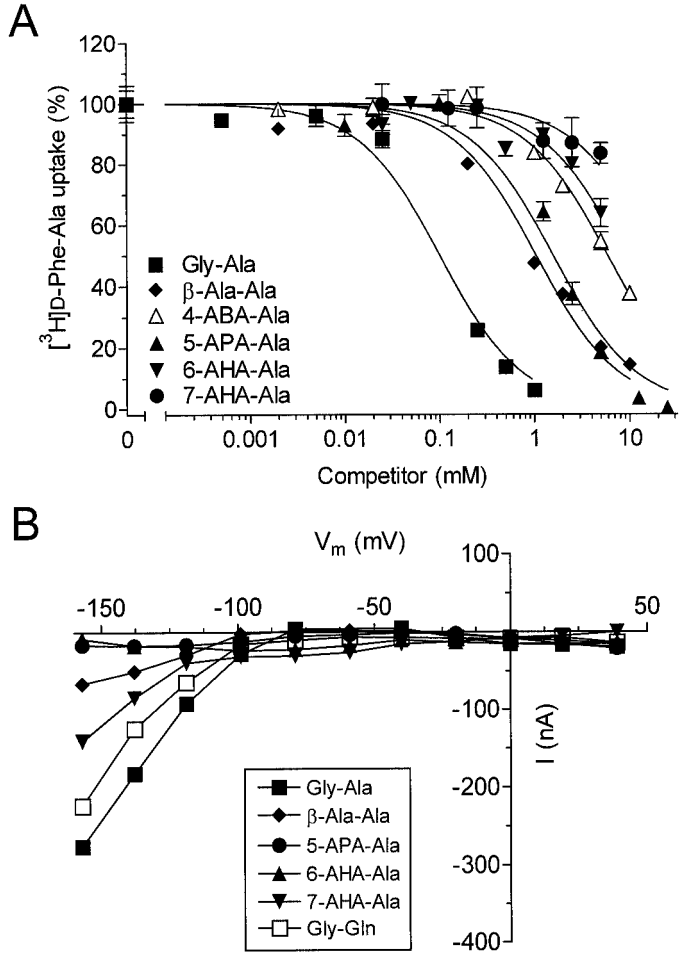


Fig. 2. Interaction of alanyl-ϖ-amino acid dipeptides with the H^+/peptide cotransporter PEPT2. A, uptake of [^{3}H]D-Phe-Ala (2 μCi/ml) into *P. pastoris* cells expressing PEPT2 was measured at pH 6.5 in the presence of increasing concentrations (0.001–25 mM) of alanyl-ϖ-amino acid dipeptides after a 15-min incubation and was nomalized against the optical density (OD) of cells. Uptake measured in the absence of competitors (301 ± 19 pmol/15 min/OD) was taken as 100%. B, steady-state I-V relationships were measured by the two-electrode voltage-clamp technique in oocytes expressing PEPT2 superfused with modified Barth's solution at pH 6.5 and 5 mM Gly-Gln or the corresponding alanyl-ϖ-amino acid derivatives. The membrane potential was stepped symmetrically to the test potentials shown, and substrate-dependent currents were obtained as the difference measured in the absence and presence of 5 mM substrate.

a conformation in which the terminal COO^- group is accommodated in a proper orientation with the required distance between the amide bond and the binding site for the C-terminal carboxyl group, as in native di- and tripeptides.

However, it remains to be answered whether a C-terminal carboxylic function is required at all for substrate recognition. Recent findings demonstrated that amino acid arylamides completely lacking a terminal carboxylic group serve as substrates for PEPT1 (Borner et al., 1998). To probe whether this also applies to PEPT2, we used a set of *para*-substituted alanine-anilides having the structures provided in Fig. 4A. As demonstrated in Fig. 4B, alanine-arylamides were able to compete for the uptake of the radiolabeled dipeptide [^{3}H]D-Phe-Ala by PEPT2 in a specific manner. Ala-anilide itself revealed a K_i value of 0.13 ± 0.04 mM (Table 2), which is comparable with the affinities of standard dipeptides. The interaction affinity of alanine-arylamides with PEPT2 could be increased gradually by introducing a methoxycarbonyl, a chloro group, or a nitro group in the *para*- position of the phenyl ring (Fig. 4B, Table 2). The Ala-4-nitroanilide showed a 16-times greater affinity to PEPT2 with a K_i value of 0.008 ± 0.001 mM than Ala-anilide. In contrast, the introduction of a carboxylic group led to a dramatic loss in affinity to 2.93 ± 0.87 mM. Because the *para*-substituents at the phenyl ring moiety of the Ala-anilide derivatives differ in their physicochemical properties such as hydrophobicity, bulkiness, or electronic (inductive and mesomeric) features (Table 2), these parameters might explain the quite impressive differences in affinity. Whereas no reasonable correlation between affinities and the aromatic substituent constants describing steric or hydrophobic properties could be found, a strong relationship was obtained when comparing the substrate affinities of the different Ala-arylamides with the Swain-Lupton parameter *F* (Fig. 4D) as an electronic field descriptor of substituents at the phenyl ring (Hansch et al., 1973).

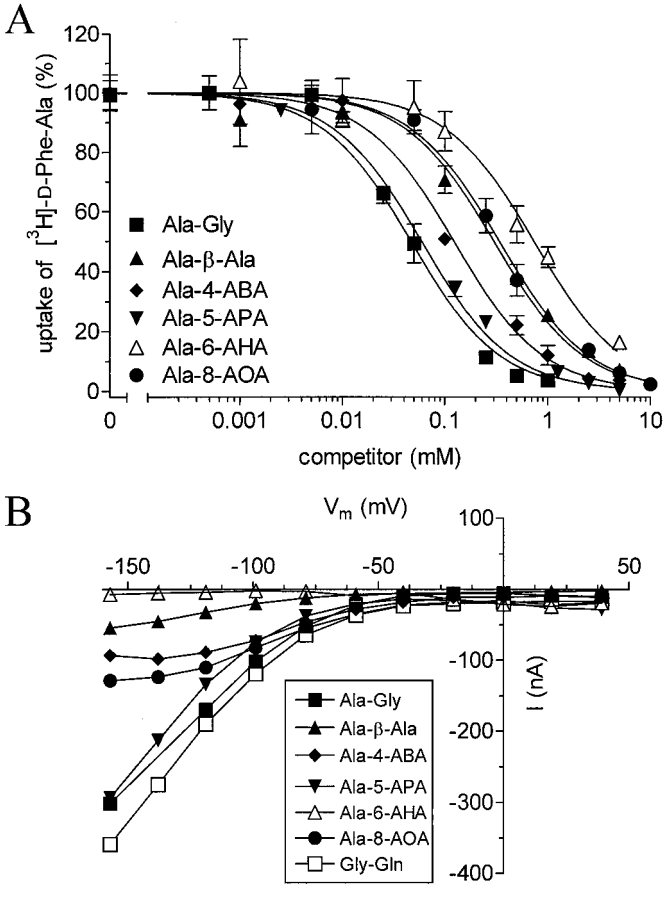


Fig. 3. Interaction of ϖ-amino acyl-alanine derivatives with the H^+/peptide cotransporter PEPT2. A, uptake of [^{3}H]D-Phe-Ala (2 μCi/ml) into *P. pastoris* cells expressing PEPT2 was measured at pH 6.5 in the presence of increasing concentrations (0.001–5 mM) of ϖ-amino acyl-alanine dipeptides after a 15-min incubation and was normalized against the optical density (OD) of cells. Uptake measured in the absence of competitors (301 ± 19 pmol/15 min/OD) was taken as 100%. B, steady-state I-V relationships were measured by the two-electrode voltage-clamp technique in oocytes expressing PEPT2 superfused with modified Barth's solution at pH 6.5 and 5 mM Gly-Gln or the corresponding ϖ-amino acyl-alanines. Substrate-dependent currents were obtained as the difference measured in the absence and presence of 5 mM substrate.

When inspecting the results from electrophysiology, it becomes obvious that substrate affinity and transport are independent read-outs of peptide transporter function. Only two compounds among the tested alanine-arylamides (Ala-anilide and Ala-4-chloroanilide) generated significant inward currents in oocytes expressing PEPT2. At a concentration of 5 mM, Ala-anilide evoked currents that were 114% of that generated by 5 mM of the standard dipeptide Gly-Gln, and the current-voltage relationship of Ala-anilide (Fig. 4C) showed no difference with I-V recordings of a normal PEPT2 substrate. Whereas the *para*-nitro derivative of Ala-anilide displayed the highest affinity (0.008 ± 0.001 mM), it is not transported electrogenically by PEPT2 (Fig. 4C, Table 2). However, electrogenic transport occurs when a chloro group is placed at the same position (K_i = 0.02 ± 0.002 mM; 58% $I_{Gly\text{-}Gln}$). Substitution of the *para*- position by a carboxylic group almost abolishes the interaction with the transporter (K_i = 2.9 ± 0.87 mM), but blocking this group by esterification, which results in an even more bulky substituent, increases the affinity almost 100-fold (K_i = 0.03 ± 0.01 mM) without regaining the capability for electrogenic transport of this compound.

Although Ala-4-nitroanilide possessed the highest affinity, it did not produce any significant inward current in PEPT2-expressing oocytes. Moreover, at a concentration of 100 μM, Ala-4-nitroanilide completely abolished the current evoked by 5 mM Gly-Gln (data not shown). This indicates that Ala-4-nitroanilide is a high-affinity inhibitor of PEPT2.

When the COO^- group of Ala-4-aminobenzoic acid was moved from the *para*- to the *meta*- position, substrate affinity increased almost 50-fold to 0.056 ± 0.003 mM and transport currents yielded 54% of $I_{Gly\text{-}Gln}$, which are characteristics similar to that of native di- or tripeptides (Fig. 5). Ala-3-aminobenzoic acid can, by its structure, be considered a perfect tripeptide mimetic with the same affinity constant and transport rate as trialanine (K_i = 0.23 ± 0.06 mM, 76% $I_{Gly\text{-}Gln}$; data not shown). When the carboxylic group attached to the phenyl ring was moved to the *ortho*- position, the affinity increased only 5-fold compared with alanine-4-aminobenzoic acid, but even at saturating substrate concentrations, no transport currents were recorded.

Discussion

Defining the minimal structural features in substrates for binding and transport by PEPT2 is important for understanding the protein structure and for the development of

peptidomimetic drugs or prodrugs that can use PEPT2 for uptake into cells expressing this carrier protein. In contrast to PEPT1, PEPT2 failed to transport ϖ-amino fatty acids such as 5-APA. However, the introduction of a carbonyl group into the backbone of 5-APA to obtain 5-amino-4-oxopentanoic acid yielded a substrate with normal affinity and electrogenic transport properties. This establishes that this carbonyl function plays an essential role in substrate recognition by PEPT2. The data obtained for ϖ-amino acyl-alanine dipeptides demonstrate the specific requirements of PEPT2 with respect to the optimal positioning of the free amino function relative to the backbone carbonyl group. Although it has already been known that a free terminal amino group is important for high-affinity renal peptide transport (Daniel et al., 1992), we demonstrate here that the terminal nitrogen atom and the identified essential backbone carbonyl group should be separated by the distance of one or at most two methylene groups from each other to enable high-affinity interaction and transport of a substrate. The marked reduction of affinity by shifting the amino group from α to β position has recently also been observed in the case of rat PEPT2 (Terada et al., 2000).

By applying the alanyl-ϖ-amino acid dipeptides, we also obtained detailed information on the importance of the positioning of the terminal carboxylic group for interaction with PEPT2. Although partly similar in chain length, the tested compounds are more flexible in conformation than tri- or tetrapeptides. Tripeptides, as well as dipeptides, are transported by PEPT2 (Boll et al., 1996; Döring et al., 1997). It is obvious that for high-affinity and electrogenic transport, a second peptide bond is not required, and even in the case of substances that, formally seen, exceed the chain length of a tripeptide, good binding and transport properties can be observed.

More striking is the finding that Ala-anilides carrying different substituents show a wide range of affinities and quite different transport characteristics. Analysis of the importance of the various *para*-substituents attached to the phenyl ring showed that higher F values corresponding to the Swain-Lupton constants correlated with lower K_i values and increased affinity for binding to PEPT2, suggesting that the electronic density at the aromatic ring system is differently affected by the inductive and mesomeric properties of the *para*-substituents; this translates into quite large differences in affinities of the Ala-anilides. The varying electronic densities may affect binding to PEPT2, either by a direct inter-

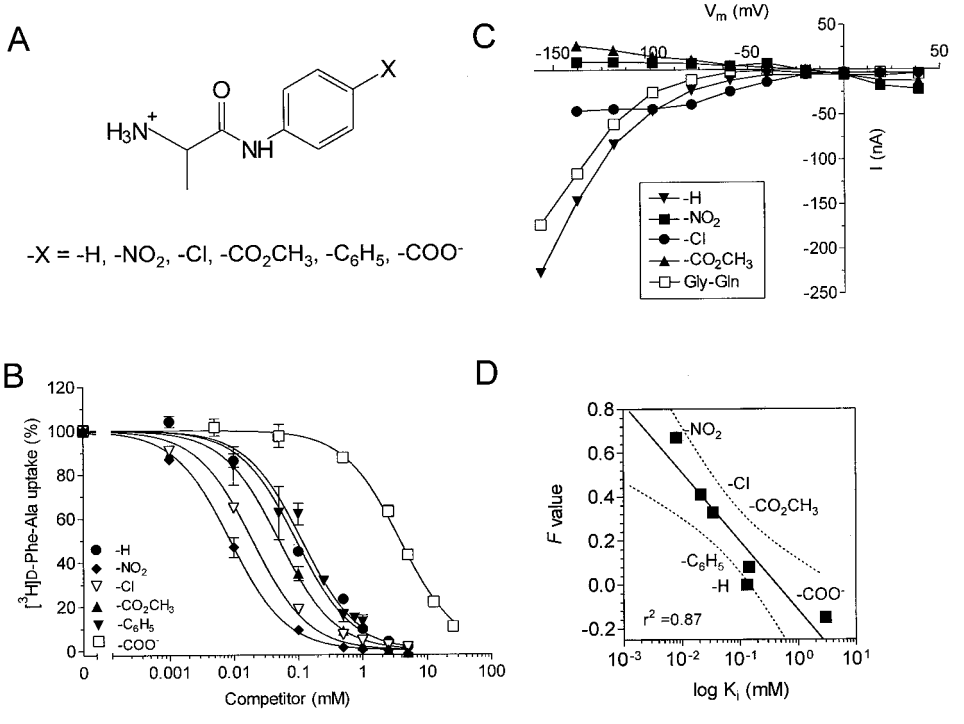


Fig. 4. Interaction of alanine-arylamides with the H^+/peptide cotransporter PEPT2. A, structures of alanine-arylamides. -H, Ala-anilide; $-NO_2$, Ala-4-nitroanilide; -Cl, Ala-4-chloroanilide; $-CO_2CH_3$, Ala-4-aminobenzoic acid methylester; $-C_6H_5$, Ala-4-phenylanilide; $-COO^-$, Ala-4-aminobenzoic acid. B, uptake of [^{3}H]D-Phe-Ala (2 μCi/ml) into *P. pastoris* cells expressing PEPT2 was measured at pH 6.5 in the presence of increasing concentrations (0.001–25 mM) of alanine-arylamides after 15-min incubation. Uptake measured in the absence of competitors (301 ± 19 pmol/15 min/OD) was taken as 100%. C, steady-state I-V relationships were measured by the two-electrode voltage-clamp technique in oocytes expressing PEPT2 superfused with modified Barth's solution at pH 6.5 and 5 mM Gly-Gln or the corresponding alanine-arylamides. The membrane potential was stepped to the test potentials shown, and substrate-dependent currents were obtained as the difference measured in the absence and presence of 5 mM substrate. D, relationship between the affinity constants (K_i) of the alanine-arylamides interacting with PEPT2 and the F-values as aromatic substituent constants. The dashed line indicates the 95% confidence interval of the linear regression line.

TABLE 2

Properties of alanine-arylamides interacting with PEPT2 and the role of aromatic substituent constants

Apparent K_i values ± S.E.M. were calculated from IC_{50} values derived by nonlinear regression analysis of the data shown in Fig. 4B. Currents were taken from the recordings of the I-V relationships shown in Fig. 4C representing the current evoked by 5 mM of the tested compound compared with that of 5 mM of Gly-Gln at a membrane potential of −100 mV. The aromatic substituent constants F (electronic), π (lipophilic), and M_R (steric, molar refraction) were taken from Hansch et al. (1973).

Compound	-X	F	π	M_R	K_i	$I_{Gly\text{-}Gln}$
					mM	%
Ala-anilide	-H	0	0	1.03	0.13 ± 0.04	114
Ala-4-nitroanilide	$-NO_2$	0.67	−0.28	7.36	0.008 ± 0.001	0
Ala-4-chloroanilide	-Cl	0.41	0.71	6.03	0.02 ± 0.002	58
Ala-4-aminobenzoic acid-methyl ester	$-CO_2CH_3$	0.33	−0.01	12.87	0.03 ± 0.01	0
Ala-4-phenylanilide	$-C_6H_5$	0.08	1.96	25.36	0.14 ± 0.03	0
Ala-4-aminobenzoic acid	$-COO^-$	−0.15	−4.36	6.05	2.93 ± 0.87	0

action of the ring system with amino acid residues within the substrate-binding pocket of the protein or by a transmission of the electrogenic effects along the phenyl ring system onto the adjacent essential carbonyl function, altering its relative charge for interaction with the transporter.

The finding that Ala-4-nitroanilide displays a high-affinity interaction with PEPT2 but is itself not transported into the cells is surprising because data obtained with Caco-2 cells and *X. laevis* oocytes expressing the intestinal peptide transporter isoform PEPT1 demonstrated that Ala-4-nitroanilide is transported electrogenically (Borner et al., 1998). This establishes that despite the similarities in substrate recognition by PEPT1 and PEPT2, there are major differences regarding the ability to transport this class of compounds electrogenically.

Taken together, our results suggest that for the transport of the Ala-anilide derivatives, the *para*-substituent needs to be rather small and uncharged, whereas a negatively charged group in this fixed position at the rigid phenyl ring prevents any interaction with the substrate-binding pocket.

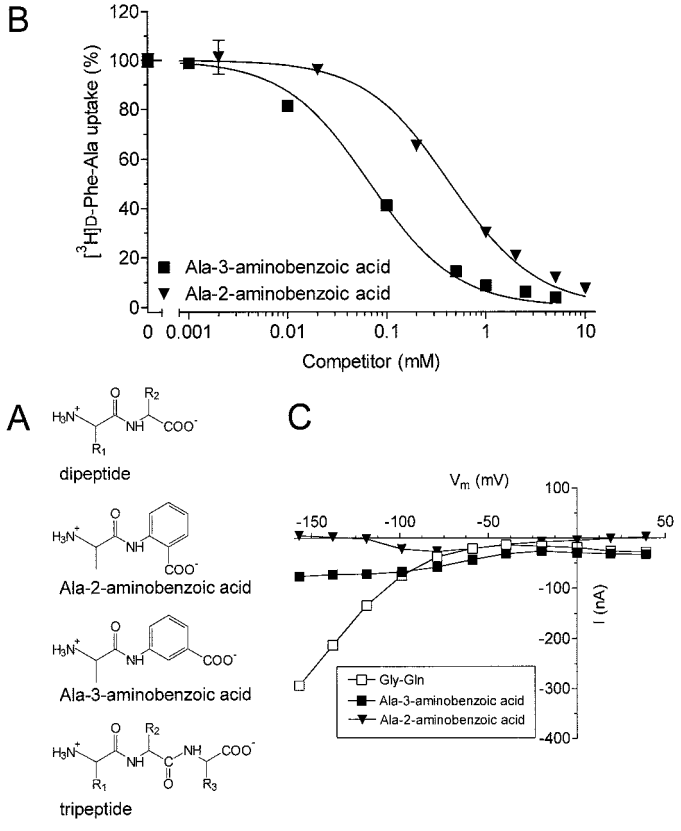


Fig. 5. Characterization of the interaction of Ala-3-aminobenzoic acid and Ala-2-aminobenzoic acid with the H^+/peptide cotransporter PEPT2. A, structure of Ala-2-aminobenzoic acid and Ala-3-aminobenzoic acid compared with the structure of di- and tripeptides. B, uptake of [^{3}H]D-Phe-Ala (2 μCi/ml) into *P. pastoris* cells expressing PEPT2 was measured at pH 6.5 in the presence of increasing concentrations (0.001–10 mM) of Ala-3-aminobenzoic acid and Ala-2-aminobenzoic acid after a 15-min incubation and was normalized against the optical density (OD) of cells. Uptake measured in the absence of competitors (301 ± 19 pmol/15 min/OD) was taken as 100%. C, steady-state I-V relationships were measured by the two-electrode voltage-clamp technique in oocytes expressing PEPT2 superfused with modified Barth's solution at pH 6.5 and 5 mM Gly-Gln or Ala-2/3-aminobenzoic acid. Substrate-dependent currents were obtained as the difference measured in the absence and presence of 5 mM substrate.

Because the COO^- group could electrostatically interact with amino acid side chains of the transporter protein at this position, we may have identified a critical functional protein domain in PEPT2 that accommodates only structures that allow hydrophobic interactions.

However, we also show here that substrates with identical N-terminal structures (alanine) but with terminal carboxylic groups attached to ϖ-amino acids of different chain length cannot only bind with high affinity; they are transported, too. The major difference between these compounds and the Ala-4-aminobenzoic acid is, of course, the alkyl spacer of the ϖ-amino acids, which possesses conformational flexibility allowing the COO^- group to be accommodated in a proper orientation within the substrate-binding domain. In the more rigid alanine-4-aminobenzoic acid, the COO^- group is sterically fixed and therefore probably prevents substrate binding within an obviously narrowly defined domain of the binding pocket. To target this domain in view of its specific requirements, we synthesized two additional alanine-aminobenzoic acid derivatives with the carboxylic acid substituent now provided in either *ortho*- or *meta*- position of the phenyl ring and determined the affinities and transport currents. The observed dramatic alterations in substrate affinity just by changing the spatial position of the COO^- substituent at the phenyl ring clearly identifies a very important region within the substrate-binding pocket of PEPT2 that can discriminate those substrates from being bound or not. Even more important is the observation that only the *meta*-substituted form allows electrogenic transport. This seems best explained by the fact that only in this structure, the COO^- group is located in a similar sterical position and distance to the essential N-terminal nitrogen and the required backbone carbonyl function, as in the terminal COO^- group in native tripeptides (Fig. 5A). We also observed that blocking the COO^- group in the *meta*- position by esterification (Ala-3-aminobenzoic acid methyl ester) retains the compound's high affinity for PEPT2 but prevents its electrogenic transport (K_i = 0.19 ± 0.06 mM, 5% $I_{Gly\text{-}Gln}$; data not shown). This further emphasizes the importance of the nature and sterical position of the substrate's COO^- group for binding and transport.

Translocation by PEPT2 of substrates that carry a terminal carboxylic group in addition to a free amino terminus and the backbone carbonyl group is only possible if the COO^- group comes into a narrowly defined spatial position within the protein-binding pocket, regardless of the overall affinity of the substrate. This is an important finding because these compounds now can be used in combination with single-site mutants of PEPT2 to identify the critical—most likely positively charged—amino acid residue in the substrate-binding region of PEPT2 that obviously controls the conformational change in the protein necessary for substrate proton translocation.

In summary, we show that there are major differences between PEPT2 and the intestinal isoform PEPT1, not only with respect to substrate affinity, but more importantly in view of the requirements for electrogenic transport. By rational design of novel amino acid anilides and modified dipeptides consisting of an N- or C-terminal α-amino acid and an ϖ-amino acid in the opposite position, the minimal structural and chemical requirements for substrate recognition and transport by PEPT2 have been defined in terms of the essential backbone length, important functional groups, and their

relative spatial location within the substrate-binding domain of PEPT2.

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