Minimal Molecular Determinants of Substrates for Recognition by the Intestinal Peptide Transporter*

(Received for publication, May 1, 1998, and in revised form, June 29, 1998)

Frank Döring, Jutta Will, Salah Amasheh‡‡, Wolfgang Claus‡, Hubertus Ahlbrecht‡, and Hannelore Daniel

From the Molecular Nutrition Center, Institute of Nutritional Science, Wilhelmstrasse 20, the Institute of Organic Chemistry, Heinrich Buff-Ring 58, and the Institute of Animal Physiology, Wartweg 95, University of Giessen, D-35392 Giessen, Germany

Proton-dependent electrogenic transporters for di- and triptides have been identified in bacteria, fungi, plants, and mammalian cells. They all show sequence-independent transport of all possible di- and triptides as well as a variety of peptidomimetics. We used the mammalian intestinal peptide transporter PEPT1 as a model to define the molecular basis for its multisubstrate specificity. By employing computational analysis of possible substrate conformations in combination with transport assays using transgenic yeast cells and Xenopus laevis oocytes expressing PEPT1, the minimal structural requirements for substrate binding and transport were determined. Based on a series of medium chain fatty acids bearing an amino group as a head group (ω-ω-amino fatty acids, ω-AFA), we show that electrogenic transport by PEPT1 requires as a minimum the two ionized head groups separated by at least four methylene groups. Consequently, a > 500 pm < 630 pm distance between the two charged centers (carboxylic carbon and amino nitrogen) is sufficient for substrate recognition and transport. Removal of either the amino group or the carbonyl group in ω-AFA maintained the affinity of the compound for interaction with the transporter but abolished the capability for electrogenic transport. Additional groups in the ω-AFA backbone that provide more hydrogen bonding sites appear to increase substrate affinity but are not essential. The information provided here does (a) explain the capability of the peptide carrier for sequence-independent transport of thousands of different substrates and (b) set the molecular basis for a rational drug design to increase the absorption of peptide-based drugs mediated by PEPT1.

Dipeptides, triptides, as well as a number of peptide-like drugs are rapidly taken up into intestinal epithelial cells by a specific apical peptide transporter encoded by the PEPT1 gene. The cDNAs of intestinal transporters of different species have been cloned from cDNA libraries (1–4), and the proteins have been characterized with respect to their operational mode by expression in Xenopus oocytes, HeLa cells, and more recently in the methyloctrophic yeast Pichia pastoris (5). At the amino acid level the PEPT1† proteins show significant homologies to peptide transporters isolated from bacteria, fungi, and plants that all belong to the PTR family of proton-dependent peptide transporters (6).

The gene products are plasma membrane carrier proteins that catalyze electrogenic uphill peptide transport by coupling of substrate translocation to the movement of H3O+ with the transmembrane electrochemical proton gradient providing the driving force (7). The physiological role of PEPT1 lies in the absorption of peptide bound amino acids from the intestinal tract after their release by enzymatic breakdown of dietary or endogenous proteins. In addition, the high availability of orally active peptide-based drugs such as almost all aminopephalosporin antibiotics, ACE-inhibitors like captopril, or peptidase inhibitors like bestatin results from their active transport mediated by PEPT1 (2, 7).

Preliminary studies (1, 2, 8–10) on the substrate specificity of PEPT1 indicated that this transporter, like its nonmammalian counterparts, transports almost all possible dipeptides, triptides, and numerous peptidomimetics. Free amino acids and tetrapeptides appear not to be accepted as substrates. Although transport of peptides and peptidomimetics occurs in a stereospecific manner (10), the transporter discriminates possible substrates only by differences in affinity for binding and/or maximal transport capacity. Based on the naturally occurring amino acids provided either as L- or D-amino enantiomers and by the huge number of different peptide-like xenobiotics, almost 1 × 106 potential substrates can be identified. Considering the wide distribution of this novel class of solute transporters throughout nature and their nutritional as well as pharmacological importance, identification of the minimal structural determinants of substrates affecting their affinity and capability for transport would greatly advance our understanding of these carrier proteins.

As a starting point, we employed the transgenic yeast system to screen a large variety of different compounds to identify a common structural motif that determines the affinity for the binding site of PEPT1. From these studies, it became obvious that the existence of a zwitterionic amino acid functionality separated by a distinct molecular distance was the most important feature. We therefore rationalized that PEPT1 should be able to interact with ω-amino fatty acids (ω-AFA) and con-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed: Prof. of Biochemistry of Nutrition, University of Giessen, Institute of Animal Physiology, Wilhelmstrasse 20, D-35392 Giessen, Germany. Tel.: 641-99-39042; Fax: 641-99-39049; E-mail: hannelore.danie@ernaehrung.uni-giessen.de.
‡ Recipient of the H. Wilhelm Schaumann Stiftung.
‡‡ To whom correspondence should be addressed: Prof. of Biochemistry of Nutrition, University of Giessen, Institute of Animal Physiology, Wilhelmstrasse 20, D-35392 Giessen, Germany. Tel.: 641-99-39042; Fax: 641-99-39049; E-mail: hannelore.danie@ernaehrung.uni-giessen.de.

† The abbreviations used are: PEPT1, rabbit intestinal peptide transporter; SGLT1, human intestinal sodium glucose transporter; PTR, peptide transporters; ω-AFA, ω-amino fatty acids; 4-ABA, 4-amino-butanolic acid; 5-APA, 5-amino-pentanoic acid; 6-ADA, 6-amino-heptanoic acid; 7-AAA, 7-amino-heptanoic acids; 8-AAAA, 8-amino-oc-tanolic acid; 11-AAA, 11-amino-decanolic acid; 2-AAA, 2-amino-oc-tanolic acid; GABA, γ-amino-butanolic acid; N-α-Z-lysine, N-α-benzoyloxycarbonyl-lysine, N-α-Z-arginine, N-α-benzoyloxycarbonyl-arginine; PPB, potassium phosphate buffer; MES, 4-morpholineethanesulfonic acid.
sequentially studied binding and transport of a series of ω-AFA. Because the amino fatty acids represent simple structures, a modeling of their possible conformations by semiempirical and 

ab initio methods and taking unspecific solvation by water into account could be performed.

The capability of PEPT1 for transport of ω-AFA was demonstrated in two different expression systems by using a radiolabeled ω-AFA as well as by measuring substrate-induced inward currents in voltage clamped Xenopus oocytes expressing the carrier. Employing the ω-AFA in combination with the modeling of these novel substrates that possess only two functional groups and a hydrocarbon backbone, we have set the basis for defining the minimal molecular requirements for substrate transport by PEPT1.

**EXPERIMENTAL PROCEDURES**

Materials—ω-AFA and other chemicals were obtained from Sigma (Deisenhofen, Germany). Custom-synthesized [H-(D)-phenylalanine-(L)-alanine ([D]-D-Phe-Ala) with a specific activity of 9 Ci mmol⁻¹ was purchased from Zeneca (Billingham, UK). [3-14C]-6-amino-hexanoic acid ([14C]-6-AHA), with a specific activity of 55 mCi mmol⁻¹, was obtained from BioTrend (Köln, Germany). [3-3H]-methyl-[3H]-D-glucose ([3H]-D-MG) with a specific activity of 86.7 Ci mmol⁻¹ was obtained from DuPont (Dreieich, Germany).

P. pastoris Strains and Induction of PEPT1 Expression in Yeast—The P. pastoris strain was cultured as described in the manual Version E of the Pichia expression kit (Invitrogen, San Diego). Construction of the Pichia strain expressing the rabbit PEPT1 and the isogenic control strain (GS-pPIC3L) has been described previously (5). In the transgenic yeast strain GS-PEPT1, the PEPT1-cDNA was placed under the transcriptional control of the AOX1 promoter, which is inducible by methanol. To induce PEPT1 expression, cells were initially grown in a Pichia strain (GS-pPIC3L) has been described previously (5). In the transgenic yeast strain GS-PEPT1, the PEPT1-cDNA was placed under the transcriptional control of the AOX1 promoter, which is inducible by methanol. To induce PEPT1 expression, cells were initially grown in a

**RESULTS**

Interaction and Transport of ω-Amino Fatty Acids by PEPT1—Based on initial observations during screening of potential substrates, we hypothesized that amino fatty acids bearing an amino group as a head group (ω-amino fatty acids, ω-AFA) could serve as substrates of PEPT1. By using a series of ω-AFA possessing 3–10 CH₃ units, we demonstrate here that these compounds indeed interact with the substrate binding site of the transporter. In P. pastoris, cells expressing PEPT1 uptake of [3H]-D-Phe-Ala was dose dependently inhibited by the ω-AFA when the compounds contained four or more CH₃ units in the backbone (Fig. 1). In the case of 4-ABA (or GABA), there was no noticeable affinity. The EC₅₀ values derived from competition curves with [3H]-D-Phe-Ala serving as a substrate ranged for the different ω-AFA between 0.30 ± 0.04 mM and 1.14 ± 0.06 mM (Table I). The apparent affinities for the ω-AFA found are very similar to those obtained for di- and tripeptides when assayed under identical experimental conditions (5).

Because competition with a dipeptide for binding at the substrate binding site does not establish that a compound is indeed transported, we employed [14C]-6-AHA as a representative structure to assess its transport characteristics in Pichia cells mediated by PEPT1. As shown in Fig. 1b, [14C]-6-AHA was taken up into the transgenic yeast cells but not into the control cells. Uptake of [14C]-6-AHA as a function of substrate concentration displayed saturation kinetics mediated by a single transport site (Fig. 1b, inset). The apparent Kᵦₐₜ value for 6-AHA influx was 0.92 ± 0.07 mM, and the EC₅₀ value for 6-Phe-Ala serving as the competitor was 0.84 ± 0.12 mM. To demonstrate that the transport of ω-AFA by PEPT1 is independent from the expression system used, we additionally employed oocytes expressing PEPT1. As shown in Fig. 1c, the EC₅₀ values for inhibition of influx of [3H]-D-Phe-Ala into oocytes by 6-AHA (0.98 ± 0.05 mM) and 8-AOA (1.27 ± 0.08 mM) are similar to those obtained in the yeast expression system. [14C]-6-AHA influx into oocytes expressing PEPT1 as a function of substrate concentration also displayed saturation kinetics (Fig. 1d) with an apparent Kᵦₐₜ value of 1.21 ± 0.32 mM. Based on these observations obtained in two different heterologous expression systems, we conclude that ω-AFAs with more than 3 CH₃ units in the backbone are recognized by the substrate binding site of PEPT1 and interact with affinities that are comparable with those of dipeptides.

To investigate whether the different ω-AFA are transported electrogenically by PEPT1, substrate-induced inward currents were measured using the 2-electrode voltage clamp setup. When a PEPT1-expressing oocyte in the voltage clamp mode was perfused with 2.5 mM ω-AFA, positive inward currents were obtained for all substrates except for 4-ABA (Fig. 2a). Whereas currents induced by some of the ω-AFAs were not as high as that induced by 2.5 mM Gly-Gln in the same oocyte, 8-AOA elicited a current of almost identical value. Moreover, when 6-AHA- and 8-AOA-mediated currents were determined as a function of membrane potential, we obtained I-V curves that are characteristic for PEPT1 and almost identical to those starting from gas phase geometries as obtained with the AM1 method. The energy of solvation by water was modeled by using the polarizable continuum model (13) implemented as the SCIPCM method into the GAUSSIAN 94 program package (14). All computations were performed with an IBM RS/6000 cluster of the computing center of the Justus-Liebig-University of Giessen.

**Calculations and Statistics**—All calculations (linear as well as nonlinear regression analysis) were performed by using PRISM (GraphPad, Los Angeles, CA). For each variable, at least two independent experiments (8–10 oocytes, 2 yeast preparations) were carried out. Data are given as the mean ± S.E. Significance of differences between the uptake rates and constants calculated were determined by a nonpaired t test.
cases with one component (phatic chains of transport). We therefore investigated if compounds with all the question of whether both head groups are required for amino and carboxyl group serve as substrates addresses amines inhibited 3H-D-Phe-Ala influx into PEPT1-expressing (fatty acids), were able to interact with PEPT1. As demonstrated by competition studies, both the fatty acids and the amines inhibited 3H-D-Phe-Ala influx into PEPT1-expressing P. pastoris cells in a dose-dependent manner. The EC50 values obtained (Table I) ranged between 0.37 ± 0.02 mM (octylamine) and 6.19 ± 0.08 mM (hexylamine). In the case of the fatty acids, EC50 values of 1.36 ± 0.07 and 2.75 ± 0.07 mM were obtained. The C8 compounds therefore displayed a 2–15-fold higher affinity than the corresponding C6 compounds. To assess whether the amines and fatty acids also elicited inward currents in oocytes expressing PEPT1, cells were superfused with 2.5 mM octylamine or octanoic acid in the voltage clamp mode. In the same oocyte in which perfusion with 2.5 mM Gly-Gln induced significant inward currents (170 nA at −60 mV), the amines and the fatty acids failed to induce measurable inward currents (Fig. 2b). This suggested that the compounds can interact with PEPT1 but cannot be transported electrogenically. To demonstrate that the amines and fatty acids inhibited PEPT1 specifically and inhibition is not caused via nonspecific interaction with the cell membrane, we used the sodium-dependent glucose transporter SGLT1 as a control.

Fig. 1. Characteristics of uptake of dipeptides and ω-amino fatty acid into P. pastoris and oocytes expressing PEPT1. a, uptake of 3H-D-Phe-Ala (2 μCi/ml) into P. pastoris cells expressing PEPT1 was measured for 30 min of incubation at pH 7.0 in the presence of increasing concentrations (0.001–50 mM) of 4-ABA ( ), 5-APS ( ), and 11-AUA ( ). Transport rates of 3H-D-Phe-Ala into control cells (GS-pPIC3L) were subtracted. Data are presented as the mean ± S.E. μ for the residual uptake in the presence of competitors (percent of control uptake; 34.09 ± 2.89 pmol/1 OD−1·30 min−1). The EC50 values were derived by the least-squares method based on a competition curve with one component and r2 in all cases ≥ 0.98. b, influx of 14C-6-AHA (2 μCi/ml) into the P. pastoris control cells ( ) or cells expressing PEPT1 ( ) was measured over 30 min of incubation at pH 7.0 in the presence of increasing concentrations of unlabeled substrate (0.001–10 mM). Uptake rates represent the mean ± S.E. μ of two independent experiments performed in triplicate. Inset, net uptake rates transformed according to Eadie-Hofstee. Kinetic constants were derived by linear regression analysis by the least-squares method. c, influx of 3H-D-Phe-Ala (10 μCi/ml) into oocytes expressing PEPT1 was measured for 30 min of incubation at pH 6.0 in the absence and the presence of increasing concentrations (0.001–20 mM) of 6-AHA ( ) and 8-AOA ( ). Uptake rates in oocytes injected with water were subtracted. Data are shown as means ± S.E. μ for residual transport activity (percent of control) as measured in 8–10 oocytes per condition and are representative for at least two experiments. The control value for uptake of 3H-D-Phe-Ala was 5.68 ± 0.51 pmol/oocyte·30 min−1. The EC50 values were derived by the least-squares method based on a competition curve with one component (r2 in all cases ≥ 0.95). d, transport activity in Xenopus oocytes expressing PEPT1 was measured for 30 min of incubation at pH 6.0 in the presence of 14C-AHA (10 μCi/ml) and increasing concentrations of unlabeled substrate (0.001–10 mM). Uptake rates in oocytes injected with water were subtracted. Uptake rates are given as the mean ± S.E. μ for 8–10 oocytes per condition and are representative for at least two similar experiments. Inset, kinetics of transport after transformation according to Eadie-Hofstee to allow kinetic constants to be derived by linear regression analysis by the least-squares method (r2 = 0.96).
Affinity of selected compounds for inhibition of \(^3\)H-n-Phe-Ala influx into \(P.\) pastoris cells expressing PEPT1

Uptake of \(^3\)H-n-Phe-Ala (2 \(\mu\)Ci \(\cdot\) ml \(^{-1}\)) into the \(P.\) pastoris cells expressing PEPT1 (1 OD) was measured for 30 min of incubation at pH 7.0 or at the pH indicated in the presence of increasing concentrations (0.001–50 mM) of competitors. Uptake rates of \(^3\)H-n-Phe-Ala into control cells (GS-pPIC3L) were subtracted. The control value for uptake of \(^3\)H-n-Phe-Ala was 34.9 \pm 2.89 pmol \(\cdot\) 1 OD \(^{-1}\) \(\cdot\) 30 min \(^{-1}\). EC\(_{50}\) values represent the mean \pm S.E. of two independent experiments performed in triplicate. EC\(_{50}\) values were derived by the least-squares method based on a competition curve with one component and \(r^2\) for regression was in all cases \(\geq 0.98\).

| Compound                        | EC\(_{50}\) value |
|---------------------------------|------------------|
| \(\omega\)-Amino fatty acids    |                  |
| 4-APA (NH\(_2\)-(CH\(_2\))\(_4\)-COO) | >50              |
| 5-APA (NH\(_2\)-(CH\(_2\))\(_5\)-COO) | 1.14 \pm 0.06    |
| 6-AHA (NH\(_2\)-(CH\(_2\))\(_5\)-COO) | 0.73 \pm 0.07    |
| 7-AHA (NH\(_2\)-(CH\(_2\))\(_6\)-COO) | 1.09 \pm 0.03    |
| 8-AOA (NH\(_2\)-(CH\(_2\))\(_6\)-COO) | 0.30 \pm 0.04    |
| 11-AUA (NH\(_2\)-(CH\(_2\))\(_9\)-COO) | 0.58 \pm 0.03    |
| Amines and fatty acids          |                  |
| Hexylamine (NH\(_2\)-(CH\(_2\))\(_5\)-CH\(_3\)) | 6.19 \pm 0.08    |
| Octylamine (NH\(_2\)-(CH\(_2\))\(_6\)-CH\(_3\)) | 0.37 \pm 0.02    |
| Hexanoic acid (CH\(_2\)-(CH\(_2\))\(_5\)-COO) | 2.75 \pm 0.07    |
| Octanoic acid (CH\(_2\)-(CH\(_2\))\(_6\)-COO) | 1.36 \pm 0.07    |
| Other compounds                 |                  |
| 5-Amino-4-oxo-pentanoic acid    | 0.27 \pm 0.03    |
| 2-Amino-octanoic acid           | >50              |
| Lysine (pH 7.0)                 | 4.89 \pm 0.22    |
| Lysine (pH 8.0)                 | 2.07 \pm 0.05    |
| Arginine (pH 7.0)               | 14.98 \pm 0.10   |
| Arginine (pH 8.0)               | 8.14 \pm 0.08    |

Octanoic acid inhibited SGLT1-mediated \(^3\)H-methyl-D-glucose influx into oocytes. Taken together, the medium chain amines and fatty acids obviously interact specifically with PEPT1 but do not show electrogentic transport.

That amino acids are not transported by the epithelial peptide transporters has been established in numerous studies employing radiolabeled amino acids as well as by using electrophysiological methods (1, 2, 8). Based on our observations with the \(\omega\)-AFA, one would assume that at least the dibasic amino acids lysine and arginine interact with PEPT1. Both possess the right backbone length (\(\geq 4\) CH\(_2\) groups) and the head groups to mimic the \(\omega\)-AFA, i.e. an amino group in the side chain and the carboxyl group bound to the \(\alpha\)-carbon. However, both amino acids carry an additional \(\alpha\)-amino group close to the carboxyl group, one could rationalize that this specific configuration prevents lysine and arginine from interacting with PEPT1. To test this hypothesis, we used lysine and arginine derivatives with protected \(\alpha\)-amino groups (N-\(\alpha\)-Z-L-lysine, N-\(\alpha\)-Z-L-arginine) as inhibitors of \(^3\)H-n-Phe-Ala influx into the transgenic \(P.\) ichia cells. At a concentration of 5 mM, both amino acid derivatives significantly (\(p < 0.001\)) reduced \(^3\)H-n-Phe-Ala uptake by 69.7 \pm 5.8\% (N-\(\alpha\)-Z-L-lysine) and 48.6 \pm 5.5\% (N-\(\alpha\)-Z-L-arginine). The free amino acids reduced dipeptide influx at 5 mM by 48.1 \pm 3.5\% (lysine) and by only 11.8 \pm 4.4\% (arginine).

In addition to the use of derivatized amino acids, we compared lysine and arginine interaction with PEPT1 at pH 7.0 and 8.0. Because of different \(pK_a\) values of the amino groups, the \(\alpha\)-amino group becomes significantly more deprotonated at pH 8.0 than the amino group in the side chain. As shown in Table I, increasing pH from 7.0 to 8.0 decreased the EC\(_{50}\) value for lysine inhibition of dipeptide influx from 4.89 \pm 0.22 to 2.07 \pm 0.05 mM and the EC\(_{50}\) value for arginine from 14.98 \pm 0.10 to 8.14 \pm 0.08 mM. In comparison, the affinity of zwitterionic substrates remains generally unaffected by these changes in pH as shown previously (2, 8). This suggested that the positive charge of the \(\alpha\)-amino group may indeed impair the affinity of the amino acids for interaction with PEPT1. To mimic the configuration of the amino and carboxyl group at the \(\alpha\)-carbon as given in amino acids, we used 2-amino-octanoic acid.

**Table I** Affinity of selected compounds for inhibition of \(^3\)H-n-Phe-Ala influx into \(P.\) pastoris cells expressing PEPT1

| Compound                        | EC\(_{50}\) value |
|---------------------------------|------------------|
| \(\omega\)-Amino fatty acids    |                  |
| 4-APA (NH\(_2\)-(CH\(_2\))\(_4\)-COO) | >50              |
| 5-APA (NH\(_2\)-(CH\(_2\))\(_5\)-COO) | 1.14 \pm 0.06    |
| 6-AHA (NH\(_2\)-(CH\(_2\))\(_5\)-COO) | 0.73 \pm 0.07    |
| 7-AHA (NH\(_2\)-(CH\(_2\))\(_6\)-COO) | 1.09 \pm 0.03    |
| 8-AOA (NH\(_2\)-(CH\(_2\))\(_6\)-COO) | 0.30 \pm 0.04    |
| 11-AUA (NH\(_2\)-(CH\(_2\))\(_9\)-COO) | 0.58 \pm 0.03    |

**Figure 2.** Recordings of substrate-induced inward currents and I-V relationships in Xenopus oocytes expressing PEPT1. a, substrate-induced inward currents in oocytes expressing PEPT1. Oocytes were clamped to –60 mV, and inward currents were measured by superfusion at pH 6.0 in the presence of 2.5 mM Gly-Gln, \(\omega\)-AFA, amines, or fatty acids. Control oocytes (water-injected) did not show any response in inward current to the addition of above compounds. b, steady-state I-V relationships were measured by the 2-electrode voltage clamp technique in oocytes expressing PEPT1 superfused with buffer pH 6.0 and 2.5 mM \(\omega\)-AFA. 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 2.5 mM substrate. Data are presented as means \(\pm\) S.E. \(\mu\) for repeated measurements in two batches of oocytes.

**Table II** Uptake of \(^3\)H-methyl-D-glucose into Xenopus oocytes expressing SGLT1 in the presence of selected compounds

| Compound                        | Uptake [% of control] |
|---------------------------------|-----------------------|
| None                            | 100.0 \pm 7.7         |
| 5-Amino-4-oxo-pentanoic acid    | 12.0 \pm 3.1\*        |
| 0.5 mM Phlorizin                | 5.5 \pm 1.2\*         |
| 8-AOA                           | 115.2 \pm 10.3        |
| Octylamine                      | 95.0 \pm 4.3          |
| Octanoic acid                   | 109.1 \pm 21.5        |

\* Significantly different when compared with the control (non-paired \(t\)-test).
The all-anti-periplanar conformations of the neutral (a) and the zwitterionic (b) forms of 4-ABA and 5-APA as well as the geometries of the cyclic zwitterionic forms (c and d) were optimized by energy minimization calculations based on the different methods and models given in the table. The predicted different conformers of 5-APA are shown in Fig. 3.

### TABLE III
Geometry optimization calculations for 4-ABA and 5-APA employing different models

| Compound  | AM1        | SM2-AM1     | SCIPC/6-31+G*//6-31+G* |
|-----------|------------|-------------|------------------------|
| 4-ABA     |            |             |                        |
| Open (a)  | $-117.7$   | 0.0         | $-124.1$               |
| Open (b)  | $-37.8$    | 83.9        | $-115.9$               |
| Cyclic (c)| $-70.8$    | 40.9        | $-113.5$               |
| Cyclic (d)| $-72.0$    | 39.7        | $-114.1$               |
| 5-APA     |            |             |                        |
| Open (a)  | $-118.7$   | 0.0         | $-130.7$               |
| Open (b)  | $-34.0$    | 84.7        | $-123.3$               |
| Cyclic (c)| $-83.0$    | 35.7        | $-119.4$               |
| Cyclic (d)| $-80.5$    | 38.2        | $-119.4$               |

| Compound  | $E_{int}^a$ | $k^b$ | $E_{rel}^a$ | $E_{rel}^a$ |
|-----------|-------------|-------|-------------|-------------|
| 4-ABA     |             |       |             |             |
| Open (a)  |             |       |             |             |
| Open (b)  |             |       |             |             |
| Cyclic (c)|             |       |             |             |
| Cyclic (d)|             |       |             |             |
| 5-APA     |             |       |             |             |
| Open (a)  |             |       |             |             |
| Open (b)  |             |       |             |             |
| Cyclic (c)|             |       |             |             |
| Cyclic (d)|             |       |             |             |

$a$ kcal/mol.

The predicted conformations for 5-APA found on the semiempirical level are presented in Fig. 3. However, on the ab initio level, the cyclic zwitterionic form of 5-APA (Fig. 3d) appeared not to be at a stationary point. By optimization, it changed to the cyclic form (c) but with a more twisted conformation, although energetically comparable. When the solvation by water was taken into account using the SM2-AM1/SM2-AM1 or the SCIPC/6-31+G*//6-31+G* model, the energetic gap between the cyclic and the open motifs was strongly reduced. As expected, both models show that the open zwitterionic form is even more favored because of its higher dipole moment that is more stabilized by solvation. These observations are in accordance with recent ab initio calculations on an artificial creatinine receptor with the option of possessing different pyridinium phenolbenzate structures (20). Here too, as more polar the environment was, the more stable was the form with a larger charge separation and a higher dipole moment. A further specific solvation by hydrogen bonding, not taken into account here, should even more favor the open zwitterionic form of the compound.

Based on our calculations, we may conclude that cyclic and open conformers of ω-AFA may exist simultaneously, but in the water environment, the open zwitterionic forms are expected to be energetically favored.

Next, we calculated the distances between the amino group and the carboxyl group centers of 4-ABA and 5-APA for both the open and cyclic conformers. As shown in Table IV, the distances calculated by the different methods are remarkably similar, especially for the open conformers designated as b. In this case, even solvation as modeled by the SM2 method did not reveal any significant effects on distance. For the cyclic conformers, some geometry changes occurred during optimization, but more importantly, as given by the values for c, all three models showed no marked differences between 4-ABA and 5-APA. For the open conformers however, the distance between the centers increased drastically from about 500 pm in the case of 4-ABA and to more than 630 pm in the case of 5-APA. Taking into account that 4-ABA did not interact with PEPT1 whereas 5-APA did, the present model analysis strongly suggests that the difference in the intramolecular distance between the functional group centers for the open conformers is a key determinant for substrate recognition.

### DISCUSSION

A number of solute transporters are designated as multispecific with respect to substrate recognition. Well documented and characterized examples are organic anion and cation transporters in kidney (21) or multidrug transporters of the MDR and MRP classes in mammalian cells (22) and bacteria (23). A broad substrate specificity is also found in procaryotic and...
eucaryotic peptide transporters of the PTR family. All family members appear to transport sequence-independent zwitterionic as well as mono- and polyvalent-charged peptides and peptidomimetics covering a molecular mass range of around 150 to 600 Da (6, 7, 24). The term multispecific, frequently applied to these carriers, simply states that there is a substantial lack of information on structural features and particularly on the conformation by which a substrate is exposed to the transporters substrate binding domain. We used the mammalian intestinal peptide transporter PEPT1 as a model for the PTR family to determine the minimal structural requirements and possible conformations of a substrate allowing the interaction with the substrate binding site and translocation across the cell membrane.

Here we show for the first time that the minimal configuration of a substrate is determined by only a very few structural elements. Demonstration that ω-AFA with 4–10 CH₂ units in the backbone bind to PEPT1 and are transported in an electrogenic fashion clearly establishes that the presence of a peptide bond in a substrate is not a structural requirement. Although previous studies using modified peptides (25, 26) demonstrated that the replacement of the peptide bond by a ketomethylene or thioxo function still retains the affinity of these dipeptide mimetics for interaction with PEPT1, our studies establish that the complete removal of the peptide bond as well as of the side chains is tolerated without a significant loss in affinity. The apparent affinities as determined for selected ω-AFA in the competition assays as well as by the influx of radiolabeled 6-AHA and electrophysiology are very similar to those of representative dipeptides or tripeptides. Moreover, characteristic inward currents and I-V relationships strongly suggest that ω-AFA are translocated in an identical mode to dipeptides, e.g. by a proton- and membrane potential-dependent electrogenic process.

To get a first insight into the conformational requirements of substrate recognition by PEPT1, we employed energy minimization calculations. FIG. 3. Optimized structures of 5-APA found on the semiempirical level. By semiempirical calculations (AM1 model) the structures of the neutral open (a), zwitterionic open (b), and zwitterionic cyclic (c and d) forms of 5-APA is given. The cyclic conformation as given in panel c contains one intramolecular hydrogen bond, whereas cyclic form with a more twisted ring conformation (d) possesses two hydrogen bonds.

### Table IV

| Method | 4-ABA, open (b) | 5-APA, open (b) | 4-ABA, cyclic (c) | 5-APA, cyclic (c) |
|--------|----------------|----------------|-----------------|-----------------|
|        | r_N..C | r_N..O<sub>syn</sub> | r_N..O<sub>anti</sub> | r_N..C | r_N..O<sub>syn</sub> | r_N..O<sub>anti</sub> |
| AM1    | 498    | 494            | 611             | 630            | 737            | 637             |
| SM2    | 504    | 520            | 617             | 632            | 739            | 657             |
| ab initio | 501    | 492            | 610             | 639            | 738            | 649             |
| AM1    | 315    | 258            | 431             | 320            | 244            | 441             |
| SM2    | 341    | 287            | 468             | 331            | 256            | 454             |
| ab initio | 328    | 248            | 448             | 337            | 248            | 454             |
zation calculations for the model compounds 4-ABA and 5-APA. They appeared to be particularly suitable because 4-ABA did not display a noticeable affinity and was not transported, whereas 5-APA showed significant affinity and electrogenic transport. The calculations revealed that the $\omega$-AFA may exist as multiple conformers in water. Although the different forms showed similar minimal energies, there was a marked difference between 4-ABA and 5-APA in the zwitterionic open forms with respect to the intramolecular distance between the centers of the ionic head groups. Therefore, the length of the separating aliphatic chain within the open all-antiperiplanar conformers (Fig. 3b) emerged as the key difference and suggests that at least a separation by four methylene groups corresponding to a distance of the polar centers (carboxylic carbon-ammonium nitrogen) of 500–630 pm is a minimal requirement for substrate interaction and transport by PEPT1.

The surprising finding that $\omega$-AFA with extended backbone of up to 10 CH$_2$ units are bound and transported by PEPT1 addresses the question of how these compounds are presented to the binding site of PEPT1 on the basis of conformation. This is an important question with respect to the general backbone length of peptide substrates since the affinity for interaction of peptides with PEPT1 is drastically reduced when the backbone length is extended over three amino acid residues (1, 2). Based on the calculated energies of the different conformers of the short chain $\omega$-AFAs, it can be expected that there is also no strong preference for one conformation in the medium chain $\omega$-AFA which by twisting of the carbon chain may easily adopt a conformation fitting into the required distance of the polar centers needed for binding to PEPT1. In contrast to the $\omega$-AFA, peptides possessing more than three amino acids are considerably less flexible because of the peptide bonds and appear by size restrictions to be excluded from the substrate binding domain of PEPT1.

As mentioned above, our studies establish that a peptide bond is not a structural requirement in a PEPT1 substrate. However, very recently it has been demonstrated that PEPT1 selectively binds and transports only the trans conformer of a dipeptide or dipeptide derivative (26). This suggested that the cis or trans bond structure is a crucial recognition parameter that seemingly contradicts our present results. However, it has to be proposed that it may be not the conformation of the peptide-bond per se that is important but that concomitant changes in the spatial location and separation of the head groups is the key factor. In dipeptides presented as cis peptide-bond conformers, the head groups do approach each other by twisting along the main bond axis and the predicted backbone changes in the spatial location and separation of the head groups is the key factor. In dipeptides presented as cis peptide-bond conformers, the head groups do approach each other by twisting along the main bond axis and the predicted backbone changes in the spatial location and separation of the head groups cannot be reached. According to this model, the cis bond dipeptide substrates would be excluded as too small to match the interacting groups in the substrate binding domain.

Whereas $\omega$-AFA with $\simeq$ 4 CH$_2$ units displayed significant affinities (1.2 mM) for binding and electrogenic transport, additional ionizable groups in close proximity to the carboxyl group impaired substrate interaction. The effect of an additional charged amino group on affinity was established by use of dibasic amino acids and their derivatives as well as by using 2-amino octanoic acid. When the two oppositely charged groups were attached to the same carbon unit, the compounds affinity decreased drastically. These observations are important for refining substrate recognition but may also explain why PEPT1, at least under physiological pH conditions, does not contribute to lysine and arginine absorption although both amino acids possess the two functional groups and the proper backbone length required.

From the studies employing the medium chain amines and fatty acids, it appears that binding of a ligand to PEPT1 requires only one of the ionized group whereas electrogenic transport requires both head groups. One functional group may be sufficient to anchor the substrate in the binding domain via salt-bridging interactions, but anchoring of the second, oppositely charged group at the proper molecular distance may be required for transport. However, when a positively charged group is placed in close proximity of the carboxyl group as realized in 2-AOA, even anchoring of the substrate via the carboxyl terminus is no longer possible. In contrast, other functional groups inserted at a different position into the backbone of an $\omega$-AFA can even increase the affinity for substrate interaction with PEPT1 as recently shown by using $\delta$-ALA ($\delta$-amino levulinic acid = 4-oxo-5-amino pentanoic acid). This precursor of chlorophyllsynthesis was identified as a high affinity substrate of PEPT1 and its renal counterpart PEPT2 (27). $\delta$-ALA, like our model substrates ($\omega$-AFAs), possesses only a amino-terminal and a carboxyl-terminal group separated by 4 CH$_2$ units but provides an additional carbonyl function at position 4. When the apparent affinity of this compound for interaction with PEPT1 is compared with that of 5-APA or the other $\omega$-AFAs, $\delta$-ALA was found to interact with a 4-fold higher affinity (see Table I). It therefore appears that the additional polar carbonyl function incorporated into the backbone of this $\omega$-AFA causes a significant increase in substrate affinity. Because the carbonyl function provides an additional site for potential hydrogen bonding within the substrate binding domain, similar groups that would allow additional electrostatic or hydrophobic interactions could generally affect the affinity of a particular substrate although such groups are not of overall importance for binding and transport.

In summary, by using $\omega$-AFA as model compounds, we show for the first time that substrate recognition and transport by the mammalian intestinal peptide transporter requires at a minimum only two ionized groups (i.e. amino- and carboxyl group) separated by an intramolecular spacer of 4 CH$_2$ units. Conformational analysis based on energy minimization calculations predicted the centers of the functional groups to be separated by a distance of $>500 < 635$ pm to enable binding to PEPT1 followed by transport. The present study therefore provides the first basis for understanding the versatility of di/tripeptide carriers and their sequence-independent transport of substrates. Whether these minimal requirements can be generalized and apply also to other members of the PTR family needs to be established. Regarding specifically PEPT1, the information provided here also could allow a more rational drug design for both peptide as well as nonpeptide mimetics to enable their interaction with PEPT1 and consequently increase their intestinal absorption.

Acknowledgment—We thank M. A. Hediger for providing the cDNA of nSGLT1.

REFERENCES

1. Fei, Y.-J., Kanai, Y., Nussberger, S., Ganapathy, V., Leibach, F. H., Romero, M. P., Singh, S. K., Boren, W. P. & Hediger, M. A. (1994) Nature 368, 563–566.
2. Boll, M., Markovich, D., Weber, W.-M., Korte, H., Daniel, H. & Murer, H. (1994) J. Physiol. 429, 146–149.
3. Liang, R., Fei, Y.-J., Prasad, D., Ramamoorthy, S., Han, H., Yang-Feng, T. L., Hediger, M. A., Ganapathy, V. & Leibach, F. H. (1995) J. Biol. Chem. 270, 6456–6463.
4. Saito, H., Okuda, M., Terada, T., Sasaki, S. & Inui, K. J. (1995) J. Pharmacol. Exp. Ther. 273, 1631–1637.
5. Doring, F., Theis, S. & Daniel, H. (1997) Biochem. Biophys. Res. Commun. 232, 656–662.
6. Steiner, H. Y., Naided, F. & Becker, J. M. (1995) Mol. Microbiol. 16, 825–834.
7. Daniel, H. (1996) J. Membr. Biol. 154, 197–203.
8. Amasheh, S., Wenzel, U., Boll, M., Dorn, D., Weber, W.-M., Claus, W. & Daniel, H. (1997) J. Membr. Biol. 155, 247–256.
9. Wenzel, U., Gebert, I., Weintraut, H., Webe, W.-M., Claus, W. & Daniel, H. (1996) J. Pharmacol. Exp. Ther. 277, 831–839.
10. Wenzel, U., Thwaites, D. T. & Daniel, H. (1995) Br. J. Pharmacol. 116,
Substrate Recognition by the Intestinal Peptide Transporter

3021–3027

11. Cramer, C. J. & Truhlar, D. G. (1991) *J. Am. Chem. Soc.* **113**, 8305–8311
12. Cramer, C. J. & Truhlar, D. G. (1992) *Science* **256**, 213–215
13. Miertus, S., Scrocco, E. & Tomasi, J. (1981) *Chem. Phys.* **55**, 117–119
14. Frisch, M. J., Trucks, G. W., Schlegel, H. B., Gill, P. M. W., Johnson, B. G., Robb, M. A., Cheeseman, J. R., Keith, T., Petersson, G. A., Montgomery, J. A., Peralta, C. Y., Ogliaro, F., Nanayakkara, A., Perst, M. A., Washietl, S., Farkas, O., Tomasi, J., Barone, V., Cossi, M., Scalmani, G., Rega, N., Petersson, G. A., Nakatsuji, H., Hada, M., Ehara, M., Toyota, K., Fukuda, R., Hasegawa, J., Ishida, M., Nakajima, T., Honda, Y., Kitao, O., Nakai, H., Klene, M., Knox, J. E., Cross, J. B., Bakken, V., Adamo, C., Jaramillo, J., Gomperts, R., Stratmann, R. E., Yazyev, O., Austin, A. J., Cammi, R., Pomelli, C., Ochterski, J. W., Martin, R. L., Morokuma, K., Farkas, O., Foresman, J. B., and O. Fox, D. J. (1995) *Gaussian 94*, Revision A.2, Gaussian, Inc., Pittsburgh, PA
15. Döring, F., Dorn, D., Bachfischer, U., Amasheh, S., Herget, M., Daniel, H. (1996) *J. Physiol.* **497**, 773–779
16. Suenram, R. D. & Lovas, F. J. (1978) *J. Mol. Spectrosc.* **72**, 372–382
17. Gordon, M. S. & Jensen, J. H. (1996) *Acc. Chem. Res.* **29**, 536–543
18. Jensen, H. J. & Gordon, M. S. (1995) *J. Am. Chem. Soc.* **117**, 8159–8170
19. Price, W. D., Jokusch, R. A. & Williams, E.-R. (1997) *J. Am. Chem. Soc.* **119**, 11988–11989
20. Craw, J. S., Copper, M. D. & Hillier, I. H. (1997) *J. Chem. Soc. Perkin Trans. I* 2, 869–871
21. Ulrich, K. J. (1997) *J. Membr. Biol.* **158**, 95–107
22. Gottesman, M. M. & Pastan, I. (1993) *Annu. Rev. Biochem.* **62**, 385–427
23. Shulman, S., Shirvan, A. & Linial, M. (1995) *Physiol. Rev.* **75**, 369–392
24. Daniel, H. & Herget, M. (1997) *Am. J. Physiol.* **273**, F1–F8
25. Enjoj, M., Hashimoto, K. & Arau, S. (1996) *Biosci. Biotechnol. Biochem.* **60**, 1893–1895
26. Brandsch, M., Thunecke, F., Kullertz, G., Schoutkowsky, M., Fischer, G. & Neubert, K. (1998) *J. Biol. Chem.* **273**, 3861–3864
27. Döring, F., Walter, J., Will, J., Foemann, M., Amasheh, S., and Daniel, H. (1998) *J. Clin. Inv.* **101**, 2761–2767