Evidence for the Absolute Conformational Specificity of the Intestinal H⁺/Peptide Symporter, PEPT1*

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This study was initiated to determine whether the intestinal H⁺/peptide symporter PEPT1 differentiates between the peptide bond conformers of substrates. We synthesized a modified dipeptide where the peptide bond is replaced by the isosteric thioxy peptide bond. The Ala-Pro derivative Ala-pgsql(CS-N)-Pro exists as a mixture of cis and trans conformations in aqueous solution and is characterized by a low cis/trans isomerization rate. The compound was recognized by PEPT1 with high affinity. The Kᵰ value of Ala-pgsql(CS-N)-Pro for the inhibition of the uptake of radiolabeled glycylsarcosine in Caco-2 cells was 0.30 ± 0.02 mM, determined in solution with 96% trans conformation. In contrast, the Kᵰ value was 0.51 ± 0.02 mM when uptake media with 62% trans conformer were used. We conclude that only the trans conformer interacts with the transport system. From our data, a significant affinity of the cis conformer at PEPT1 cannot be derived. In a second approach, conformer-specific uptake of Ala-pgsql(CS-N)-Pro was studied by analyzing the intracellular content of Caco-2 cells following transport as well as the composition of the extracellular medium using capillary electrophoresis. The percentage of trans conformer that was 62% in the uptake medium increased to 92% inside the cells. This is the first direct evidence that an H⁺/peptide cotransport system selectively binds and transports the trans conformer of a peptide derivative.

The peptide transporters expressed in the brush border membrane of the intestinal and renal epithelial cells are responsible for the absorption of oligopeptides that consist of two or three amino acids. These transporters are driven by a transmembrane proton gradient and mediate the symport of their substrates with H⁺ (1–4). In addition to their natural substrates, peptide transporters are also capable of transporting many pharmacologically active peptidomimetics (5, 6). The structural requirements for substrates to be accepted are not completely understood. However, all of the chemically diverse substrates studied so far seem to bear interacting sites correctly aligned to the transporter by sterical resemblance to the backbone of physiologically occurring di- and tripeptides (5, 6).

Therefore, in the design of pharmacologically active peptidomimetics that interact with peptide carriers, modification of the backbone will become increasingly important.

In the present investigation, we studied the influence of the substrate backbone dynamics caused by peptide bond cis/trans isomerization on the intestinal peptide transport. Both cis and trans conformers can be found in peptides and proteins due to the partial double bond character of the peptide bond. Yet, the free energy difference between cis and trans isomers is lower in the amide than in the amide bond. Therefore, among peptides made of gene-coded amino acids, a significant population of the energetically disfavored cis isomerization has been described so far only for Xaa-Pro peptide bonds (7, 8). For proteases, it has been shown that they prefer the trans conformation at and near the scissile bond of their substrates (9). On the other hand, peptidyl cis/trans isomerases utilize both cis and trans isomers of peptide and protein substrates (8). For peptide receptors, a preference either for cis conformers (10) or for all-trans conformers (11, 12) has been hypothesized. Whether peptide transporters are able to differentiate between cis and trans conformers of di- and tripeptides has not yet been shown. Measuring 4- to 12-fold lower affinities of Gly-Pro and Gly-Sar compared with Gly-Gly and Gly-Ala at the renal peptide transporter, Daniel et al. (13), however, have already discussed that isomerization at the peptide bond might be responsible. To study cis/trans conformational effects, ideally, conformers of one and the same substrate should be used. Unfortunately, it has been proven to be difficult to stabilize pure peptide bond conformers for a period sufficient for transport experiments because the cis/trans interconversion of natural Xaa-Pro dipeptides occurs at room temperature within seconds to minutes (8). A peptide therefore had to be found that is characterized by being a proteolytically stable substrate for the peptide carrier, by a sufficiently low cis/trans interconversion rate and by the possibility to vary its isomer ratio. We have chosen to synthesize a modified Ala-Pro where the peptide carbonyl oxygen is replaced by sulfur. Thioxy oligopeptides are known to be isoergic to their non-thioxylated counterparts (14). However, replacing the peptide bond by a thioxy peptide bond in Xaa-Pro peptides resulted in an up to 100-fold retardation in the cis/trans isomerization rates due to a higher barrier of rotation at the C–N bond (14, 15). Using the dipeptide derivative Ala-pgsql(CS-N)-Pro as a substrate in the present investigation, we provide first direct evidence for the conformational specificity of peptide transport.

EXPERIMENTAL PROCEDURES

Synthesis of Ala-pgsql(CS-N)-Pro—The thioxy peptide was synthesized by thioxylation of Boc-Ala-Pro-OtBu with Lawesson’s reagent (15),
deprotected using 95% trifluoroacetic acid, and characterized by \textsuperscript{1}H-,
\textsuperscript{13}C-NMR, mass spectrometry and HPCE.\textsuperscript{1}

**HPCE and NMR Analyses—**HPCE analysis was performed using a BioFocus 3000 (Bio-Rad, Germany) (14, 16). UV detection was done at 270 nm where Ala-\(\psi\)-(CS-N)-Pro shows maximal absorption. \textsuperscript{1}H and
\textsuperscript{13}C-NMR spectra were recorded on an ARX-500 spectrometer (Bruker, Germany) at 500.13 and 125.76 MHz, respectively. Assignment of signal
sets to cis and trans conformation was done using the \textsuperscript{13}C-NMR pattern of Pro\(\alpha\) and Pro\(\gamma\) signals as well as the characteristic upfield shift of the cis AlaC\(\alpha\) signal in the \textsuperscript{1}H spectra compared with the corresponding trans signal (7).

**Cell Culture and Uptake Measurements—**The human colon carcinoma cell line Caco-2 was obtained from the German Collection of Microorganisms and Cell Cultures and routinely cultured as described (17, 18). Uptake of [glycine-\textsuperscript{14}C]Gly-Sar (53 mCi/mmol, Amersham International, UK) was measured 8 days after seeding (17). The uptake buffer (1 ml) contained 25 mM Mes/Tris (pH 6.0), 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 0.8 mM MgSO\(_4\), 5 mM glucose, 20 mM [\textsuperscript{14}C]Gly-Sar, and increasing concentrations of unlabeled Ala-\(\psi\)-(CS-N)-Pro (0 to 5 mM). In a different approach, uptake of Ala-\(\psi\)-(CS-N)-Pro was measured by incubating the washed monolayers with 1 ml of uptake medium containing Ala-\(\psi\)-(CS-N)-Pro (1 mM) for 10, 30, or 60 min. After washing the cells, 1 ml of deionized water was added to each monolayer. The dishes were frozen and thawed twice, and contents were suspended with the use of a 1-ml syringe and 25-gauge needle. Centrifugation at 70,000 \(\times\) g for 10 min yielded a clear supernatant that was analyzed using HPCE.

**Data Analysis—**Results are given as means \(\pm\) S.E. (\(n = 6–9\)). Non-linear regression analysis, calculation of inhibition constants (\(K_i\)) from IC\(_{50}\) values and statistical analysis was done as described (17, 18). Areas of HPCE peaks were integrated and divided by migration time.

**RESULTS AND DISCUSSION**

**Cis/trans Ratio of Ala-\(\psi\)-(CS-N)-Pro—**Both \textsuperscript{1}H- and \textsuperscript{13}C-NMR spectra showed two sets of signals for Ala-\(\psi\)-(CS-N)-Pro, which were assigned to trans and cis isomers. Using HPCE, we were able to separate the cis/trans conformers of Ala-\(\psi\)-(CS-N)-Pro. A striking feature of Ala-\(\psi\)-(CS-N)-Pro was that immediately after dissolving in uptake buffer at pH 6.0, this compound had a trans content of 96\%, whereas only 4\% were in cis conformation (Fig. 1, solution I). After dissolving the peptide, interconversion of Ala-\(\psi\)-(CS-N)-Pro isomers proceeded slowly toward cis/trans equilibrium. After 2 days at room temperature, equilibrium with 62\% trans and 38\% cis isomers was reached (Fig. 1, solution II). Both ratios were verified by NMR analysis (Fig. 1). Such differences in cis/trans ratios between ad hoc solutions and equilibrated solutions have already been described for natural Xaa-Pro dipeptide conformers (16). The evidently low interconversion rate of Ala-\(\psi\)-(CS-N)-Pro isomers, which enabled the following experiments to be done, was confirmed by monitoring the cis/trans ratio changes after dissolution using HPCE. These measurements revealed a half-time of about 12 h for Ala-\(\psi\)-(CS-N)-Pro isomers at 30 \(^\circ\)C. Storing Ala-\(\psi\)-(CS-N)-Pro solution at \(-20\) \(^\circ\)C delayed the isomerization rate further, thereby actually “locking” the respective cis/trans ratio. Therefore, for all the following experiments, Ala-\(\psi\)-(CS-N)-Pro solutions (pH 6.0) were prepared, sterilized, and split into solution I that was kept at \(-20\) \(^\circ\)C for 2 days and solution II that was kept at 22 \(^\circ\)C for 2 days. Before each experiment, solutions were diluted to the desired substrate concentration, and the cis/trans ratio was determined by HPCE.

**Inhibition of [\textsuperscript{14}C]Gly-Sar Uptake by Ala-\(\psi\)-(CS-N)-Pro I and II—**Uptake of Gly-Sar into Caco-2 cells is driven by an inwardly directed H\textsuperscript{+} gradient (17) and mediated by a single transport system (\(K_i = 1.1 \pm 0.1\) mM), which has been identified as the low affinity, high capacity system PEPT1 present in human small intestine (17–19). We measured the ability of Ala-\(\psi\)-(CS-N)-Pro to inhibit \[\textsuperscript{14}C\]Gly-Sar uptake in dependence of the cis/trans ratio. In case of solution I, where the relative \(\text{trans}\) content was 96\% (Fig. 1), the \(K_i\) value was 0.30 \(\pm\) 0.02 mM (Fig. 2), which is comparable with those of Ala-Xaa dipeptides (17). However, the \(K_i\) value was increased by 70\% to 0.51 \(\pm\) 0.02 mM when the equilibrated Ala-\(\psi\)-(CS-N)-Pro solutions containing only 62\% trans conformer were used. Shown in Fig. 2 also is the theoretical displacement curve that results under the presumption that, at a cis/trans ratio of 38/62\%, exclusively the trans isomer competes with labeled Gly-Sar at the binding site of the transport system. The measured and the simulated displacement curves are virtually identical. This provides evidence that only the trans isomer of Ala-\(\psi\)-(CS-N)-Pro interacts with PEPT1. We cannot completely rule out at this time, however, a very low affinity (\(K_i > 40\) mM) of the cis conformer at PEPT1, but this cannot be derived from our data with any confidence.

**Intracellular Accumulation of Ala-\(\psi\)-(CS-N)-Pro—**Demonstrating the competition between trans Ala-\(\psi\)-(CS-N)-Pro and Gly-Sar at the binding site of PEPT1 does not allow the unambiguous conclusion that trans Ala-\(\psi\)-(CS-N)-Pro is transported into the cell. Therefore, in a different approach, we analyzed

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\textsuperscript{1} The abbreviations used are: HPCE, high-performance capillary electrophoresis; Gly-Sar, glycylsarcosine; Mes, 4-morpholinethanesulfonic acid.
the intracellular content of Caco-2 cells by HPCE after uptake of Ala-\(\psi\)-(CS-N)-Pro. The incubation medium contained Ala-\(\psi\)-(CS-N)-Pro (1 mM, pH 6.0) at cis/trans equilibrium (62% trans, Fig. 1, solution II). Inside the cells, however, the relative trans content increased in a time-dependent manner (Fig. 3 B-D) reaching 92 ± 0.7%. As shown in Fig. 3, B-D, the cis conformer also appeared inside the cells during incubation. Because of the long half-time for establishing the conformer-specific Peptide Transport 3863 conformational equilibrium, an increase of the cis amount due to re-equilibration inside the cells is negligible during the experiment. More likely, the minor increase in cis species was due to simple diffusion through the cell membrane. To confirm that, we repeated the experiment in the absence (outside 7.5) and presence (outside 6.0) of a pH gradient using a 30-min uptake with cell homogenates. The peptide concentration and the cis/trans ratio remained unchanged (63% trans before and 64% trans after incubation with cell homogenates).

It was of great advantage that at a detection wavelength of 270 nm, the spectral interference with signals arising from intrinsic constituents of Caco-2 cells is low. The electropherogram (Fig. 3A) shows no endogenous peaks localized at the positions where the Ala-\(\psi\)-(CS-N)-Pro signals appeared. The shift in migration time of Ala-\(\psi\)-(CS-N)-Pro seen in Fig. 3 compared with data obtained in pure buffer (Fig. 1) most likely reflects matrix effects due to the cellular material. After uptake experiments (Fig. 3, B-D) there was no additional peak except the two which we identified routinely as being cis and trans isomers of Ala-\(\psi\)-(CS-N)-Pro by adding 1 \(\mu\)l of 1 mM solution II to the sample. It should be noted that the endogenous peak preceding the trans peak is of nucleotidic rather than peptidic origin as shown by HPCE-analysis at 260–280 nm. The area of this peak also increased in homogenates of untreated control cells (sample A) after 2 days.

In a third approach, uptake of the cis/trans isomers of Ala-\(\psi\)-(CS-N)-Pro by Caco-2 monolayers (12 days in culture) was
measured by analyzing the uptake medium (solution II, 50 μM, 0.4 ml, 15–120 min). The cis amount decreased by 7% in 2 h, which corresponds well to the expected amount of simple diffusion. At the same time, however, the trans amount in the medium decreased by 34%, indicating again that only the trans conformer of Ala-ψ[CS-N]-Pro was transported.

In summary, the results of the present study combined provide unequivocal evidence that (i) isosteric backbone modifications such as thioxylation can be tolerated by PEPT1. More importantly, (ii) the intestinal peptide carrier is absolutely specific for the trans backbone conformation of Ala-ψ[CS-N]-Pro. The results strongly imply that PEPT1 though tolerating many diverse substrates is able to differentiate decisively the spatial arrangement of the chain segment bracketed by the Cα atoms of the transported oligopeptides. The efficacy of the intestinal absorption of peptides containing tertiary amide bonds compared with that of other peptides will additionally depend on the cis/trans isomerization. At this step, regulatory and metabolic processes might affect the delivery of peptides containing proline differently from that of non-proline containing oligopeptides because cellular events preceding transport such as degradation and modification of proline peptides are known to occur in an isomer-specific manner as well.

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