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ARTICLE

Transport of Free and Peptide-Bound Pyrraline at Intestinal and Renal Epithelial Cells

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Pyrraline is a quantitatively dominating glycation compound of the advanced Maillard reaction in foods and can be found in urine after consumption of pyrraline-containing food items. The purpose of this study was to investigate the transport of pyrraline and its dipeptide derivatives alanylpyrraline (Ala-Pyrr) and pyrralylalanine (Pyrr-Ala) at intestinal and renal cell lines. Pyrraline inhibited the L-[^{3}H]lysine uptake with IC_{50} values of 0.3 mM (Caco-2 cells) and 3.5 mM (OK cells), respectively, but not the uptake of [^{14}C]Gly-Sar (Caco-2 and SKPT cells). In contrast, Ala-Pyrr strongly inhibited the uptake of [^{14}C]Gly-Sar in Caco-2 and SKPT cells with IC_{50} values of 0.19 and 0.017 mM, respectively. Pyrr-Ala inhibited the carrier-mediated uptake of [^{14}C]Gly-Sar in Caco-2 and SKPT cells by 50% at concentrations of 0.03 and 0.008 mM, respectively. The transepithelial flux of peptide-bound pyrraline across Caco-2 cell monolayers was up to 15-fold higher compared to the flux of free pyrraline. We conclude that free pyrraline is not a substrate for the intestinal lysine transporter and that the absorption of dietary pyrraline occurs most likely in the form of dipeptides rather than as the free amino acid.

KEYWORDS: Glycation; Maillard reaction; AGE; pyrraline; membrane transport; intestine; kidney; absorption

INTRODUCTION

The Maillard reaction, often also termed nonenzymatic browning or glycation, is of utmost importance for the formation of flavor and color during thermal processing of food and furthermore influences the nutritional quality of stored foodstuffs. The reaction mainly occurs between reducing carbohydrates and their degradation products and the ε-amino group of lysine, the N-termini of proteins, and the guanidino group of arginine (*1*). In addition to the Amadori compounds (e.g., N^{ε}-fructosyllysine and N^{ε}-lactulosyllysine), which are predominantly formed in the early course of the reaction cascade, several structures from later stages of the reaction called "advanced glycation end products" (AGEs) have been identified in foods (*2*), among them pyrraline (6-(2-formyl-5-hydroxymethyl-1-pyrrolyl)-L-norleucin), which is generated during the reaction of 3-deoxy-D-*erythro*-hexos-2-ulose (3-deoxyglucosulose, 3-DG) with the ε-amino group of lysine (*3*). Pyrraline was first isolated by Nakayama et al. (*4*) and was shown to have low mutagenic activity (*5*) and antinutritional properties due to the inhibition of intestinal peptidases (*6*). Pyrraline, as quantified in food by amino acid analysis (*7*), can be found predominantly on sites of high thermal impact and low moisture content, like in bread crust, rusk, or crackers, in which its content may range up to 3680 mg per kg of protein. Other important sources are dried foodstuffs like milk and whey powder (*7*) and pasta products (*8*) with concentrations varying between 90 and 150 mg per kg of protein. The daily intake of AGEs (mainly pyrraline and CML) has been estimated to be 25−75 mg (*2*).

It is currently under debate whether dietary AGEs represent a risk to human health (*9*). Dietary AGEs are reported to promote oxidative stress and to aggravate the sequelae of diabetes and uremia (*10*). A reduced intake of AGEs from food is advised in order to prevent age-related diseases like diabetes (*11*, *12*), often presuming general absorption of AGEs from the diet with a consequential increase of inflammatory markers reflecting a low-grade systemic inflammation (*11*, *13*). On the other hand, no significant proof could yet be given if the accumulation in vivo of AGEs in the course of the diseases mentioned is a causative or concomitant effect (*14*).

To elicit effects within the human body, however, absorption in effective amounts across the intestinal epithelial barrier is required. In contrast to their relevance in conventional nutrition and their possible pathophysiological role, quite little is known about the "metabolic fate" of AGEs. A balance study with 18 healthy volunteers showed individual absorption and elimination properties of individual AGEs (*15*, *16*). After the consumption of a test meal containing N^{ε}-fructosyllysine, pyrraline, and pentosidine within a food matrix, between 50% and 100% of the administered protein-bound pyrraline was found as the free amino acid in the urine together with 60% of pentosidine administered as the free amino acid, while only less than 5% of

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the initially protein-bound pentosidine and N^{ε}-fructosyllysine were recovered.

Hypothetically, the compounds can cross the intestinal epithelial barrier via the paracellular route (simple diffusion) or transcellularly by diffusion, endocytotic processes, or mediation by transport proteins. Possible candidates for carrier-mediated transport are the naturally occurring carriers for amino acids, sugars, or peptides. We have shown recently that N^{ε}-carboxymethyllysine, N^{α}-hippuryl-N^{ε}-fructosyllysine, N^{α}-hippuryl-N^{ε}-carboxymethyllysine, and N^{ε}-fructosyllysine are neither transported by PEPT1 nor transported by carriers for neutral amino acids (*17*). The low transepithelial flux from the apical to the basolateral side of Caco-2 cell monolayers measured for these compounds occurred most likely by simple diffusion.

The situation might be very different for dipeptide derivatives of AGEs. In this study, we investigated whether pyrraline or the dipeptide derivatives alanylpyrraline (Ala-Pyrr) and pyrralylalanine (Pyrr-Ala) are potential substrates for lysine or peptide transporters. It has been established in recent years that at enterocytes three different transporters of the solute carrier (SLC) superfamily are able to translocate lysine across the apical membrane into the cells, namely, the systems $B^{0,+}$, $b^{0,+}$ and y^{+}. Di- and tripeptides are transported by PEPT1. This transporter is driven by a transmembrane H^{+} gradient and catalyzes the cotransport of its substrates with H^{+} into intestinal and other cells (for a review see ref *18*). PEPT1 accepts many amino acid derivatives and modified dipeptides as substrates as long as the structural requirements for substrate binding and translocation are met (for a review see ref *19*).

To study the intestinal and renal transport, pyrraline and its alanyl dipeptide derivatives were synthesized and characterized spectroscopically. Alanine was chosen as "partner" for pyrraline because it represents a common hydrophobic amino acid in food proteins. Furthermore, alanine-containing dipeptides generally are used for investigating structural requirements of peptide carriers (*20*). In competition assays versus radiolabeled lysine and Gly-Sar, their interaction with the carriers responsible for the uptake of cationic amino acids and dipeptides was determined in Caco-2, SKPT, and OK cells. Moreover, we measured the total transepithelial net flux across monolayers of the human colon cell line Caco-2 and the renal cell line OK.

MATERIALS AND METHODS

Materials. N^{α}-*tert*-Butoxycarbonyl-L-lysine (N^{α}-Boc-lysine) was obtained from Fluka (Steinheim, Germany). N^{ε}-*tert*-Butoxycarbonyl-L-alanyl-L-lysine (Boc-Ala-Lys) and N^{ε}-*tert*-butoxycarbonyl-L-lysyl-L-alanine (Boc-Lys-Ala) were purchased from Iris Biotech (Martinsried, Germany). 3-Deoxyglucosulose (3-DG) was prepared according to Henle and Bachmann (*3*). HPLC gradient grade acetonitrile was from VWR Prolabo (Leuven, Belgium) and sodium 1-heptanesulfonate from Alfa Aesar (Karlsruhe, Germany). Microcrystalline cellulose (particle size 20−160 μm) from Merck (Darmstadt, Germany) was used. DOWEX AG 50W-X8 ion-exchange resin (100−200 mesh) was from Acros (Geel, Belgium). The water used for the preparation of buffers and solutions was obtained using a Purelab plus purification system (USFilter, Ransbach-Baumbach, Germany). All other chemicals were purchased from standard suppliers and were of the highest purity available.

The human colon carcinoma cell line Caco-2 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The renal cell line SKPT-0193 Cl.2, established from isolated cells of rat proximal tubules, was provided by U. Hopfer (Case Western Reserve University, Cleveland, OH; cf. ref *21*). The opossum kidney cell line OK was provided by H. Daniel (Molecular Nutrition Unit, Technical University of Munich, Germany). Cell culture media, supplements, and trypsin solution were purchased from Life Technologies, Inc. (Karlsruhe, Germany) or PAA (Pasching, Austria). Fetal bovine serum was from Biochrom (Berlin, Germany). [Glycine-1-^{14}C]glycylsarcosine (specific radioactivity 56 mCi/mmol) and L-[4,5-^{3}H]lysine monohydrochloride (specific radioactivity 99 Ci/mmol) were obtained from GE Healthcare (Little Chalfont, U.K.). [^{14}C]Mannitol (specific radioactivity 53 mCi/mmol) was obtained from Hartmann Analytic GmbH (Braunschweig, Germany).

High Pressure Liquid Chromatography (HPLC) Analysis of Pyrraline and Pyrraline Containing Dipeptides. All analytical HPLC analyses were performed using a high pressure gradient system from Amersham Pharmacia Biotech (Uppsala, Sweden), consisting of a pump P-900 with an online degasser (Knauer, Berlin, Germany), a column oven, and a UV detector UV-900. Analytical separation of pyrraline and the respective dipeptide analogues was achieved using a polymer-based RP-18-column (PLRP-S, 100 Å, 8 μm, 250 mm × 4.6 mm, Polymer Laboratories, Darmstadt, Germany). An amount of 50 μL of the samples obtained from the cell culture experiments was injected. The column temperature was set to 30 °C, and UV detection was performed at 297 nm. The mobile phase consisted of 5 mM sodium heptanesulfonate, pH 2.0 (solvent A), and acetonitrile (solvent B). A linear gradient from 2% to 27% B in 23 min was used for the measurements of pyrraline and Ala-Pyrr containing samples (gradient A). The gradient for Pyrr-Ala samples was from 7% to 18.5% B in 35 min (gradient B). The flow rate was 1.0 mL/min. External calibration was performed with the synthesized standards.

The samples from the flux measurements were treated as follows: an amount of 50 μL of samples from the apical compartment was diluted with 450 μL of solvent A, and an amount of 50 μL of samples from the basolateral compartment was mixed with 100 μL of solvent A. The membrane samples were thawed and refrozen three times in order to completely release the cellular contents. A total of 300 μL of this solution was then added to 300 μL of solvent A.

Mass Spectrometry. For MS analyses, a PerSeptive Biosystems Mariner time-of-flight mass spectrometry instrument equipped with an electrospray ionization source (ESI-TOF-MS, Applied Biosystems, Stafford, TX) working in the positive mode was used. Calibration of the mass scale was established using a mixture of bradykinin, angiotensin I, and neurotensin.

After appropriate dilution of the samples with 1% formic acid in 50% acetonitrile, the sample was injected at a flow rate of 5 μL/min into the ESI source by a syringe pump. Spray tip potential, nozzle potential, quadrupole rf voltage, and detector voltage were set at 4812.3, 80, 1000, and 2400 V, respectively.

Nuclear Magnetic Resonance Spectrometry (NMR). ^{1}H NMR spectra were recorded on a Bruker DRX 500 instrument (Reinstetten, Germany) at 500 MHz. Deuterium oxide was used as the solvent. Proton chemical shifts are given relative to the internal HOD signal (4.70 ppm).

Elemental Analysis. Elemental analysis data were obtained on a Euro EA 3000 elemental analyzer (Eurovector, Milano, Italy).

Synthesis and Isolation of 6-(2-Formyl-5-hydroxymethyl-1-pyrrolyl)-L-norleucin (Pyrraline). The synthesis method described by Henle and Bachmann (*3*) was followed with modified isolation of the synthesis product. Then 534 mg (2.2 mmol) of Boc-Lys-OH and 1400 mg (8.7 mmol) of 3-deoxyglucosulose (3-DG) were dissolved in 6.5 mL of 0.1 N sodium acetate buffer, pH 5.0, and the pH was adjusted to 5.0 with acetic acid. The solution was mixed with 5.2 g of cellulose powder and incubated for 4 h at 70 °C in a drying oven after lyophilization. The brown powder was then extracted three times with 100 mL portions of water. The pooled extracts were concentrated to 50 mL in vacuo at 40 °C. Isolation of crude Boc-pyrraline was performed by setting the pH value to 1.0 with sulfuric acid and extracting at least 4 times each with 50 mL of diethyl ether. Black precipitates, which might appear, were always dissolved with dilute sodium hydroxide and united with the aqueous phase, the pH of which was adjusted to 1.0 with sulfuric acid. The combined organic layers were evaporated to dryness and dissolved in 1000 mL of 10% acetic acid. The solution was heated under reflux at 70 °C for 4 h in order to hydrolyze the Boc protecting group. Acetic acid was then removed by rotary evaporation. The brown residue was dissolved in 15 mL of 0.1 M pyridine/formic acid buffer, pH 3.0, and the pH adjusted to 3.0 with formic acid. The separation of pyrraline from byproduct was achieved by semipreparative ion-exchange chromatography (*4*, *22*) using a column (1.5 cm × 48 cm) of DOWEX 50 WX-8 (100−200 mesh) previously

equilibrated with 250 mL of 6 N hydrochloric acid, 250 mL of water, 250 mL of 2 N aqueous pyridine, 250 mL of water, and 250 mL of 0.1 M pyridine/formic acid buffer, pH 3.0. Pyrraline was eluted with 0.3 M pyridine/formic acid buffer, pH 3.75, at a flow rate of 0.35 mL/min. Fractions of 10 mL were collected using a fraction collector (RediFrac, Pharmacia Biotech, Uppsala, Sweden), and the presence of the product was first monitored by spotting 1 μL of the fractions on TLC plates and spraying with 0.1% ninhydrin in ethanol. Selected fractions were then diluted and analyzed by HPLC using the appropriate gradient systems described above. Pyrraline was found to elute between 130 and 250 mL. The fractions were combined, repeatedly evaporated in vacuo, and taken up in water until the smell of pyridine had become imperceptible. Finally, the residue was dissolved in a small volume of water; the solution was filtered and lyophilized to give an amorphous light-brown powder of pyrraline, which was stored at −20 °C.

Pyrraline: ESI-MS, positive mode, $[M + H]^+$ m/z 255.1; ^{1}H NMR (500 MHz, D_2O), δ [ppm] 1.29 (2H, m, Lys-H4), 1.66 (2H, m, Lys-H5), 1.76 (2H, m, Lys-H3), 3.60 (1H, t, Lys-H2), 4.20 (2H, t, Lys-H6), 4.57 (2H, s, 5-C$\underline{H}_2$OH), 6.25 (1H, d, J = 4.1 Hz, pyrrolyl-H4), 7.02 (1H, d, J = 4.2 Hz, pyrrolyl-H3), 9.23 (1H, s, 2-CHO). Elemental analysis: $C_{12}H_{18}N_2O_4$ (MW = 254.28), calcd, C 56.68%, H 7.13%, N 11.02%; found, C 55.08%, H 8.02%, N 10.66%; content = 96.7%, based on nitrogen. Yield = 119 mg (molar yield = 23.0%).

Synthesis and Isolation of Peptide-Bound Pyrraline as L-Alanyl-6-(2-formyl-5-hydroxymethyl-1-pyrrolyl)-L-norleucin (Ala-Pyrr) and 6-(2-Formyl-5-hydroxymethyl-1-pyrrolyl)-L-norleucyl-L-alanine (Pyrr-Ala). The method described above was adapted. Then 495 mg (1.6 mmol) of the Boc-protected dipeptides and 1.08 g (6.7 mmol) of 3-DG were dissolved in 4.7 mL of 0.1 N sodium acetate buffer, pH 5.0, and the pH was adjusted to 5.0 with acetic acid. After the solutions were mixed with 3.8 g of cellulose powder, the incubation and the extraction of the crude reaction product were like for pyrraline. After the first extraction step, however, the pH of the aqueous phase was adjusted to pH 4.5 and additionally extracted four times with ethyl acetate. Deprotection and separation were performed as above. Under these conditions, the modified dipeptides eluted between 400 and 650 mL.

Ala-Pyrr: ESI-MS, positive mode, $[M + H]^+$ m/z 326.2; ^{1}H NMR (500 MHz, D_2O), δ [ppm] 1.24 (2H, m, Lys-H4), 1.43 (3H, d, Ala-CH_3), 1.61 (2H, m, Lys-H5), 1.70 (2H, m, Lys-H3), 3.97 (1H, qd, Ala-H2), 4.03 (1H, dd, Lys-H2), 4.17 (2H, t, Lys-H6), 4.57 (2H, s, 5-C$\underline{H}_2$OH), 6.25 (1H, d, J = 4.1 Hz, pyrrolyl-H4), 7.01 (1H, d, J = 4.1 Hz, pyrrolyl-H3), 9.22 (1H, s, 2-CHO). Elemental analysis: $C_{15}H_{23}N_3O_5$ (MW = 325.36), calcd, C 55.37%, H 7.13%, N 12.91%; found, C 52.44%, H 6.42%, N 11.40%; content = 88.3%, based on nitrogen. Yield = 248 mg (molar yield = 42.1%).

Pyrr-Ala: ESI-MS, positive mode, $[M + H]^+$ m/z 326.2; ^{1}H NMR (500 MHz, D_2O), δ [ppm] 1.24 (3H, d, Ala-CH_3), 1.32 (2H, m, Lys-H4), 1.66 (2H, m, Lys-H5), 1.79 (2H, m, Lys-H3), 3.85 (1H, t, Lys-H2), 4.03 (1H, qd, Ala-H2), 4.21 (2H, t, Lys-H6), 4.57 (2H, s, 5-C$\underline{H}_2$OH), 6.25 (1H, d, J=4.1 Hz, pyrrolyl-H4), 7.03 (1H, d, J=4.2 Hz, pyrrolyl-H3), 9.23 (1H, s, 2-CHO). Elemental analysis: $C_{15}H_{23}N_3O_5$ (MW = 325.36), calcd, C 55.37%, H 7.13%, N 12.91%; found, C 55.06%, H 6.88%, N 12.09%; content = 93.6%, based on nitrogen. Yield = 156 mg (molar yield = 28.0%).

Cell Culture. Caco-2 cells (passage 35−99) were routinely cultured in 75 cm^2 culture flasks with minimum essential medium supplemented with 10% fetal bovine serum, gentamicin (50 μg/mL), and 1% nonessential amino acid solution at 37 °C in a humidified atmosphere with 5% CO_2 (*17*, *23*−*25*). Subconfluent cultures (90% of confluence) were treated 5 min with Dulbecco's phosphate-buffered saline followed by a 2 min incubation with trypsin solution. For most experiments, the cells were seeded in 35 mm disposable Petri dishes (Sarstedt, Nümbrecht, Germany) at a density of 0.8×10^6 cells per dish. The uptake measurements were performed on the seventh day after seeding. Protein content per dish was determined according to the Bradford method. Caco-2 cells were also cultured on permeable polycarbonate Transwell cell culture inserts (diameter 24 mm, pore size 3 μm, Costar GmbH, Bodenheim, Germany) with a cell density of 0.2×10^6 cells/filter for 21 days (*17*, *23*, *24*). The lower (receiver) compartment contained 2.6 mL of medium and the upper (donor) compartment 1.5 mL of medium. The transepithelial electrical resistance was measured at day 21 using a Millicell ERS (Millipore Intertech, Bedford, MA). Caco-2 cell monolayers reached a transepithelial electric resistance of 619 ± 23 Ω cm^2.

OK cells (passage 39−66) were cultured in Dulbecco's modified Eagle's medium/F12 nutrient mixture (1:1, v/v) supplemented with fetal bovine serum (10%, w/v), penicillin−streptomycin (1%), and glutamine (1%). OK cells were seeded in Petri dishes at a density of 0.8×10^6 cells per dish. The uptake measurements were performed on the seventh day after seeding. OK cells were also cultured in Transwell chambers (diameter 24 mm, pore size 0.4 μm, Costar GmbH, Bodenheim, Germany) with a seeding cell density of 0.4×10^6 cells/filter and a culture period of 21 days.

Culture medium for SKPT cells (passage 58−89) was Dulbecco's modified Eagle's medium/F12 nutrient mixture (1:1, v/v) supplemented with fetal bovine serum (10%, w/v), gentamicin (50 μg/mL), epidermal growth factor (10 ng/mL), insulin (4 μg/mL), dexamethasone (5 μg/mL), and apo-transferrin (5 μg/mL). SKPT cells were seeded in Petri dishes at a density of 0.8×10^6 cells per dish. The uptake measurements were performed on the fourth day after seeding (*21*, *25*).

Transport Studies. Uptake of [^{14}C]Gly-Sar in Caco-2 and SKPT cells cultured on plastic dishes was measured at room temperature as described earlier (*17*, *21*, *23*, *25*). The uptake buffer 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, [^{14}C]Gly-Sar, and unlabeled compounds at increasing concentrations. After incubation for 10 min, the cells were quickly washed four times, dissolved in Igepal CA-630 buffer, and prepared for liquid scintillation spectrometry. The nonsaturable component of [^{14}C]Gly-Sar uptake (diffusion, adherent radioactivity) determined by measuring the uptake of [^{14}C]Gly-Sar in the presence of 50 mM (Caco-2) or 20 mM (SKPT) unlabeled Gly-Sar represented 8.4% and 8.9% of the total uptake, respectively. This value was taken into account during nonlinear regression analysis of inhibition constants.

Uptake of L-[^{3}H]lysine in Caco-2 and OK cells cultured on plastic dishes was measured in the absence or presence of unlabeled compounds for 5 min. The nonsaturable component of L-[^{3}H]lysine uptake (diffusion, adherent radioactivity) determined by measuring the uptake of L-[^{3}H]-lysine in the presence of 20 mM unlabeled L-lysine represented 21% (Caco-2) and 8% (OK) of the total uptake.

Transepithelial flux of pyrraline, Ala-Pyrr, and Pyrr-Ala across Caco-2 and OK cell monolayers was measured as follows (*17*, *23*, *24*). All experiments were performed at day 21 after seeding at 37 °C in a shaking water bath. After washing the inserts with buffer (25 mM Hepes/Tris (pH 7.5), 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose), uptake was started by adding uptake buffer (pH 6.0, 1.5 mL) containing compounds (1 mM) to the donor side. At time intervals of 10, 30, 60, and 120 min, 200 μL samples were taken from the receiver compartment and replaced with fresh buffer (pH 7.5). Samples were stored until analysis. After 2 h, the filters were quickly washed four times with ice-cold uptake buffer, cut out of the plastic insert, stored in 1 mL of 10% TCA solution, and frozen.

Data Analysis. Experiments were done in duplicate or triplicate, and each experiment was repeated two to three times. Results are given as mean values ± SEM. IC_{50} values (i.e., concentration of unlabeled compounds necessary to inhibit 50% of [^{14}C]Gly-Sar or l-[^{3}H]lysine carrier-mediated uptake) were determined by nonlinear regression. Flux data were calculated after correction for the amount taken out by linear regression of appearance in the receiver well vs time.

RESULTS AND DISCUSSION

Synthesis and Analysis of Free and Peptide-Bound Pyrraline. In two previous studies, we provided evidence that nearly the complete amount of peptide-bound pyrraline administered with heated foods such as bakery products or milk can be found in the urine of healthy volunteers within 24 h (*15*, *16*). For a renal excretion, the release of pyrraline from food proteins during gastrointestinal digestion is necessary. Absorption from the chyme then requires transport of compounds across the intestinal epithelium, which in the case of amino acids and peptides can be effected by different amino acid transporters but also by the di- and tripeptide transporter PEPT1 (*18*). Since pyrraline is a known inhibitor of intestinal peptidases (*6*), it should quite likely

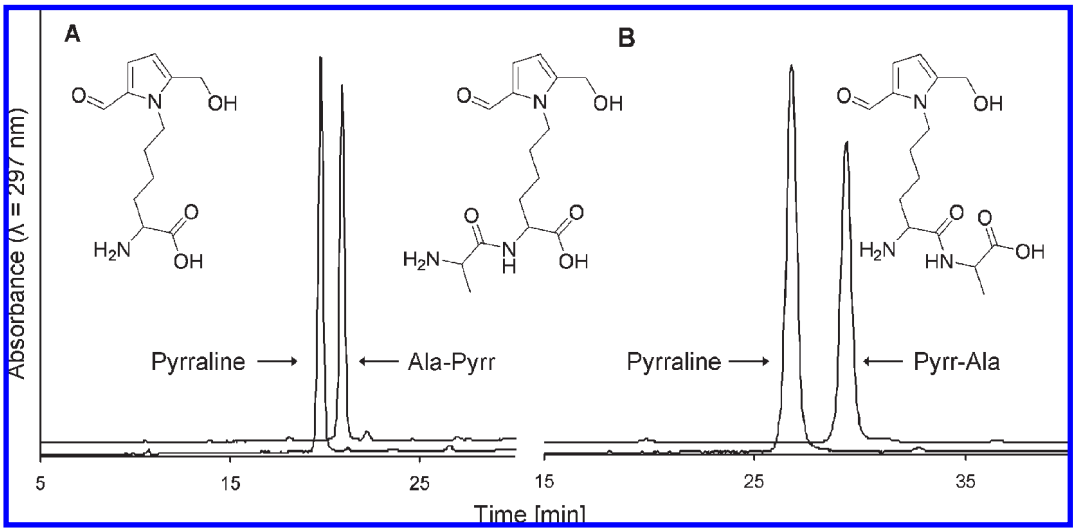

Figure 1. Structures of pyrraline and its dipeptide derivatives and HPLC chromatograms showing the purity and separation of pyrraline and Ala-Pyrr (**A**) and pyrraline and Pyrr-Ala (**B**), respectively, using two different gradient systems.

accumulate bound in small peptides. As a continuation of our studies on the nutritional physiology of early and advanced glycation compounds (*17*), we wanted to investigate how pyrraline permeates the intestinal epithelial barrier. Attention was not only paid to the free amino acid but also to peptide-bound pyrraline as well. The position of a modified amino acid in a dipeptide can be decisive for its inhibitory and transport characteristics (*18*). We therefore modified the lysyl residues in both the dipeptides Ala-Lys and Lys-Ala to pyrraline. As a prerequisite for our studies, sufficient amounts of free and peptide-bound pyrraline were needed. The synthesis protocol of Henle and Bachmann (*3*), which affords high pyrraline yields, was followed and the resulting pyrraline further purified by ion-exchange chromatography after the extraction of Boc-pyrraline from the acidified reaction mixture. The method was adapted to the protected dipeptides Boc-Ala-Lys and Boc-Lys-Ala, which were commercially available, resulting in substantial amounts of the pyrraline-modified dipeptides for the first time. **Figure 1** shows the structures and the chromatographic purity of the synthesized compounds. The identity of the compounds was checked by mass spectrometry. Results of NMR were in agreement with published data (*3*).

The analytical separation of pyrraline from its dipeptide derivatives was achieved by RP-HPLC using a polymer-based column and heptanesulfonic acid as an ion-pairing reagent (**Figure 1**). As low as 0.5 nmol/mL pyrraline and 0.8 nmol/mL Ala-Pyrr could be quantified with gradient A and 1.0 nmol/mL pyrraline and 1.3 nmol/mL Pyrr-Ala with gradient B. The intra-assay coefficient of variation was lower than 5%. The limits of quantification represent about 0.1% of the concentration applied to the apical membrane at the beginning of the flux measurements, thus enabling evaluation of quite small transport phenomena.

Interaction with Proton-Coupled Transporters for Di- and Tripeptides. We first investigated whether pyrraline, Ala-Pyrr, or Pyrr-Ala interact with the intestinal or renal H^+/peptide cotransporters PEPT1 and PEPT2. As a labeled reference substrate, we used [^{14}C]Gly-Sar (glycylsarcosine). Gly-Sar is used as reference dipeptide because it is relatively stable against intra- and intercellular enzymatic hydrolysis. Ala-Pyrr and Pyrr-Ala at a concentration of 10 mM inhibited the [^{14}C]Gly-Sar uptake into Caco-2 cells expressing PEPT1 by 94% and 91%, respectively (**Table 1**). In SKPT cells, which express the high-affinity isoform PEPT2 and, therefore, serve as standard model for PEPT2 studies, both compounds at a concentration of 2 mM inhibited [^{14}C]Gly-Sar uptake by 97% and 90%, respectively (**Table 1**). Pyrraline showed only a slight inhibition of the [^{14}C]Gly-Sar uptake in both cell lines (**Table 1**). We next performed competition experiments using increasing concentrations of the three Maillard reaction products to determine IC_{50} values for [^{14}C]-Gly-Sar uptake inhibition (**Figure 2A** and **Figure 2B**). For Ala-Pyrr, IC_{50} values of 0.19 ± 0.01 mM at Caco-2 cells (PEPT1) and 0.017 ± 0.001 mM at SKPT (PEPT2) were obtained. For Pyrr-Ala, the IC_{50} values were 0.03 ± 0.01 mM (PEPT1) and 0.008 ± 0.001 mM (PEPT2, **Table 1**). These values qualify both dipeptide derivatives according to our classification as high affinity ligands for PEPT1 as well as for PEPT2 (*19*). In contrast, pyrraline showed no affinity for the two peptide transporters (**Table 1**). For comparison, we also measured the interaction of L-lysine and the dipeptide Gly-Sar with PEPT1 and PEPT2. Whereas the amino acid L-lysine showed no affinity to PEPT1 and PEPT2, Gly-Sar represents a medium affinity PEPT1 and PEPT2 substrate with IC_{50} values of 0.74 ± 0.01 and 0.11 ± 0.01 mM, respectively (**Table 1**, **Figure 2**). We conclude that peptide-bound but not free pyrraline interacts with H^+/peptide cotransporters.

Interaction with L-Lysine Transporters. Transporters for L-lysine are possible candidates for transport of free pyrraline. Therefore, we studied whether pyrraline or its derivatives interfere with lysine uptake at Caco-2 and OK cells. OK cells do not express PEPT2. They are, however, an often used cell model for

Table 1. Inhibition of [^{14}C]Gly-Sar Uptake (10 μM) by Pyrraline, Ala-Pyrr, Pyrr-Ala, Gly-Sar, and L-Lysine in Caco-2 and SKPT Cells[a]

	Caco-2, [^{14}C]Gly-Sar uptake		SKPT, [^{14}C]Gly-Sar uptake	
compd	uptake (%)	IC_{50} (mM)	uptake (%)	IC_{50} (mM)
control	100 ± 8		100 ± 6	
Gly-Sar	15 ± 1	0.74 ± 0.01	9.4 ± 0.3	0.11 ± 0.01
L-lysine	101 ± 4		102 ± 3	
pyrraline	77 ± 2	>10 (~48)[b]	96 ± 3	>10 (~53)[b]
Ala-Pyrr	5.5 ± 0.4	0.19 ± 0.01	3.3 ± 0.6	0.017 ± 0.001
Pyrr-Ala	9.4 ± 0.2	0.03 ± 0.01	9.5 ± 1.2	0.008 ± 0.001

[a] Uptake was measured at pH 6.0 for 10 min in the absence (control) or presence of inhibitors at fixed concentrations (10 mM for Caco-2, 2 mM for SKPT) for % uptake or at increasing concentrations of unlabeled inhibitors for determination of IC_{50} values. Data are mean values ± SE (n=4). [b] IC_{50} values extrapolated beyond measurement range because of limited solubility of compounds or low inhibition.

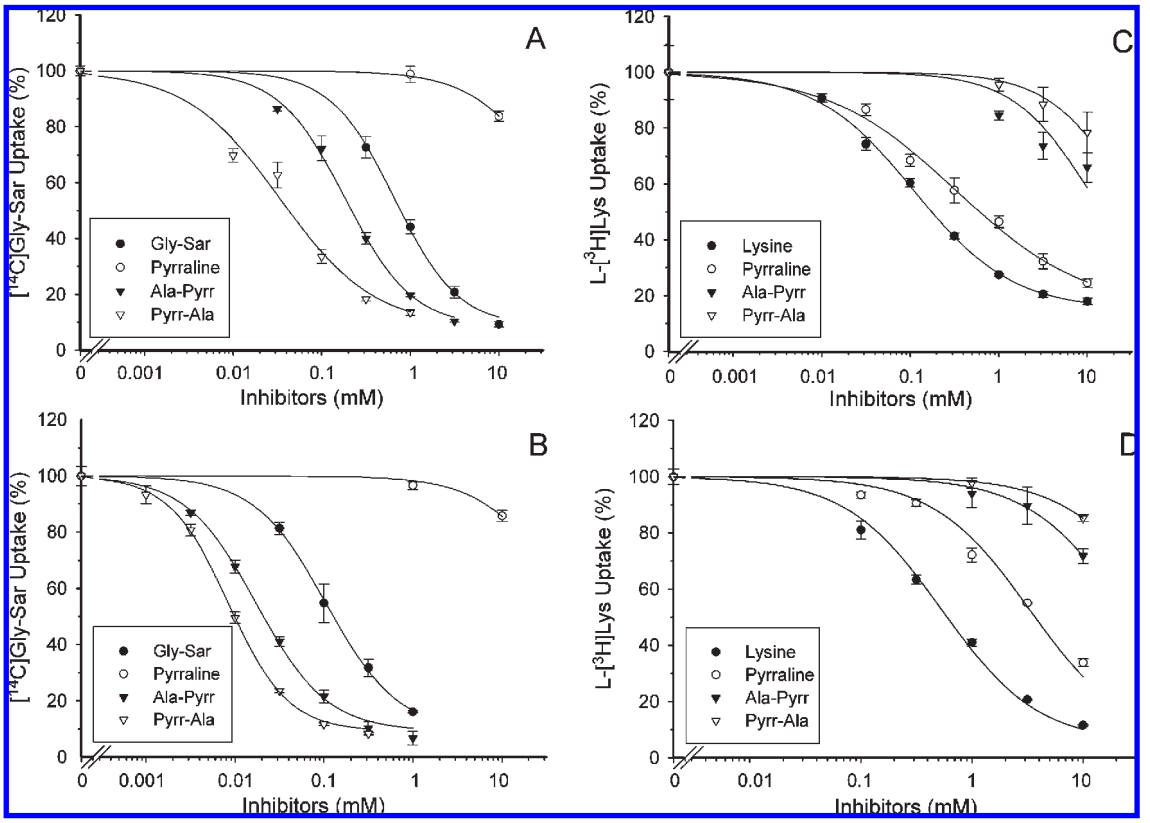

Figure 2. Inhibition of [^{14}C]Gly-Sar and L-[^{3}H]lysine uptake into Caco-2, OK, and SKPT cells by pyrraline, Ala-Pyrr, Pyrr-Ala, Gly-Sar, and L-lysine. Uptake of 10 μM [^{14}C]Gly-Sar was measured for 10 min in Caco-2 (**A**) and SKPT cells (**B**) at pH 6.0 in the absence (control) or presence of increasing concentrations of the compounds. Uptake of 2 nM L-[^{3}H]lysine was measured for 5 min in Caco-2 (**C**) and OK cells (**D**) at pH 6.0 in the absence (control) or presence of increasing concentrations of the compounds. Data are mean values ± SE, $n = 3-4$.

renal amino acid transport studies. Ala-Pyrr and Pyrr-Ala at a concentration of 10 mM only weakly inhibited the uptake of L-[^{3}H]lysine into Caco-2 and OK cells (**Table 2**). A stronger inhibition by 75% in Caco-2 cells and by 67% in OK cells was observed with free pyrraline. We determined IC_{50} values of 0.32 ± 0.04 mM (Caco-2) and 3.5 ± 0.2 mM (OK) for free pyrraline (**Table 2**, **Figure 2**). For comparison, we also measured the inhibition of L-[^{3}H]lysine uptake by unlabeled Gly-Sar and L-lysine. L-Lysine inhibited the uptake of L-[^{3}H]lysine with an IC_{50} value of 0.11 ± 0.01 mM (Caco-2) and 0.51 ± 0.09 mM (OK), whereas Gly-Sar showed no affinity to the amino acid transporter (**Table 2**, **Figure 2**). We then studied the stability of the derivatives in uptake buffer. When Ala-Pyrr and Pyrr-Ala were added to Caco-2 cells for 10 min at a concentration of 1 mM, 9.4 ± 0.3% Ala-Pyrr and 2.1 ± 0.3% Pyrr-Ala were hydrolyzed to pyrraline. Therefore, the moderate inhibition of L-[^{3}H]lysine uptake by Ala-Pyrr and Pyrr-Ala can easily be explained by free pyrraline originating from the dipeptides during incubation together with its high affinity toward the L-lysine transporter(s). We conclude that free pyrraline but not the dipeptide derivatives interacts with amino acid transporters for lysine, either as a substrate or as an inhibitor.

Transepithelial Flux of Pyrraline, Ala-Pyrr, and Pyrr-Ala. Inhibition of uptake of a transporter reference substrate does not necessarily mean that the interacting inhibitors are transported themselves. They might represent nontransported compounds with a certain affinity to the transporters. To investigate whether pyrraline and its dipeptide derivatives Ala-Pyrr and Pyrr-Ala show significant transepithelial transport, we determined their total net transepithelial flux across Caco-2 and OK cells by HPLC analysis. These experiments were done at pH 6.0 over 2 h. At the end of the flux measurements, the filters supporting the monolayers were cut out of the inserts and also analyzed.

Table 2. Inhibition of L-[^{3}H]Lysine Uptake (2 nM) by Pyrraline, Ala-Pyrr, Pyrr-Ala, L-Lysine, and Gly-Sar in Caco-2 and OK Cells[a]

	Caco-2, L-[^{3}H]lysine uptake		OK, L-[^{3}H]lysine uptake	
compd	uptake (%)	IC_{50} (mM)	uptake (%)	IC_{50} (mM)
control	100 ± 6		100 ± 4	
L-lysine	17 ± 2	0.11 ± 0.01	13 ± 1	0.51 ± 0.09
Gly-Sar	105 ± 5		104 ± 2	
pyrraline	25 ± 1	0.32 ± 0.04	33 ± 1	3.5 ± 0.2
Ala-Pyrr	66 ± 5	>10 (~11)[b]	72 ± 3	>10 (~24)[b]
Pyrr-Ala	78 ± 7	>10 (~30)[b]	93 ± 2	>10 (~54)[b]

[a] Uptake was measured at pH 6.0 for 5 min in the absence (control) or presence of inhibitors at a fixed concentration (10 mM) for % uptake or at increasing concentrations of unlabeled inhibitors for determination of IC_{50} values. Data are mean values ± SE ($n = 4$). [b] IC_{50} values extrapolated beyond measurement range because of limited solubility of compounds or low inhibition.

The results are as follows: When added at a concentration of 1 mM to the luminal (apical) side, neither Ala-Pyrr nor Pyrr-Ala was found at the basolateral (abluminal) side or in the cells. In both compartments, the substrate amounts were below the detection limit. Small amounts of free pyrraline, also added to the apical side at a concentration of 1 mM, could be detected both in the cells and in the basolateral compartment, but the flux rate was even lower than that of the space marker [^{14}C]mannitol (0.07 ± 0.02%/cm^2 h compared to 0.13 ± 0.03%/cm^2 h; **Figure 3**). The intracellular amount after 2 h was 0.59% of the total pyrraline amount applied (**Figure 3**, inset). From these data we conclude that Ala-Pyrr and Pyrr-Ala are not transported across

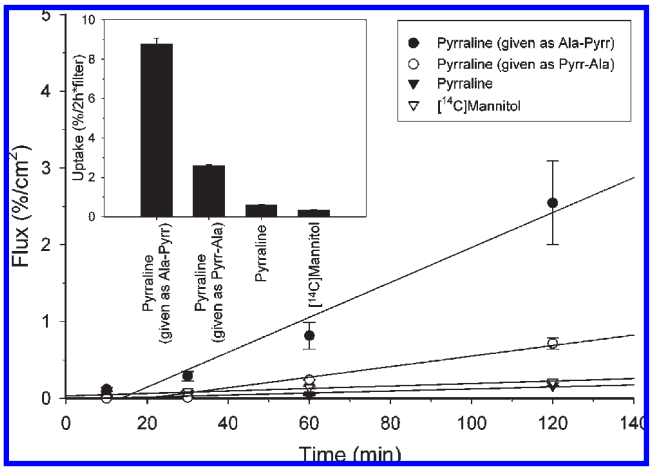

Figure 3. Transepithelial flux of pyrraline, Ala-Pyrr, Pyrr-Ala, and [^{14}C]-mannitol across Caco-2 cells. Flux was determined in the presence of 1 mM compounds and 10 μM [^{14}C]mannitol, respectively, at pH 6.0 (apical) and pH 7.5 (basolateral) over 2 h. Data are mean values ± SE, n = 3.

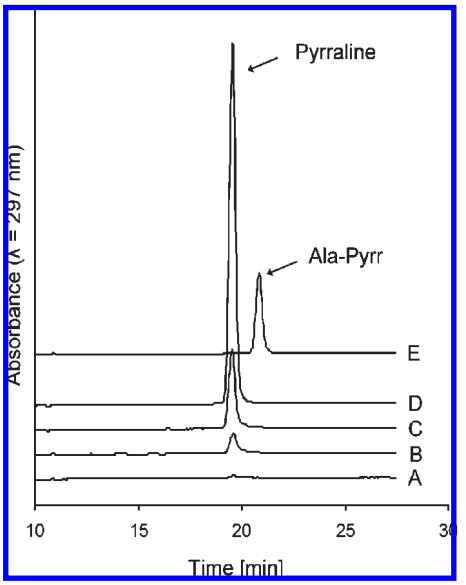

Figure 4. Appearance of pyrraline in the basolateral compartment during the flux measurement of Ala-Pyrr at Caco-2 cells 10 min (**A**), 30 min (**B**), 60 min (**C**), and 120 min (**D**) after addition of 1 mM Ala-Pyrr to the apical compartment. The intact dipeptide Ala-Pyrr (**E**, standard chromatogram) could not be detected.

Caco-2 cells in intact form. The transport of free pyrraline, even though it interacts with lysine transporting amino acid transporters, is neglectable.

The situation turned out to be completely different when we analyzed free basolateral and intracellular pyrraline concentration in samples where Ala-Pyrr and Pyrr-Ala (1 mM) had been added to the luminal compartment for 2 h. When given in the form of dipeptides, pyrraline appeared in the basolateral compartment (**Figure 4**), and high flux rates of pyrraline were obtained. In case of Ala-Pyrr, a pyrraline flux rate of 1.06 ± 0.27%/(cm^2 h) was observed (**Figure 3**). When 1 mM Pyrr-Ala was added to the cells, basolateral pyrraline was detected with a rate of 0.28 ± 0.08%/(cm^2 h) (**Figure 3**). In both cases, the flux rates were higher than the flux of the space marker [^{14}C]mannitol. Within the cells only pyrraline but not the peptide form was found (8.8% and 2.6%, respectively, **Figure 3**, inset).

We conclude that Ala-Pyrr and Pyrr-Ala, but not free pyrraline, are taken up into intestinal cells across the apical membrane by the peptide transporter PEPT1. Once inside the cells, Ala-Pyrr and Pyrr-Ala are hydrolyzed to free pyrraline and alanine. In the presence of a pH gradient, PEPT1 is able to transport its substrates uphill against a concentration gradient (*19*). Therefore, a pyrraline gradient across the basolateral membrane exists and the basolateral efflux of free pyrraline via simple diffusion will be driven by this gradient.

In order to evaluate a possible renal reabsorption of pyrraline from primary urine, we also measured the transepithelial flux of 1 mM pyrraline across OK cell monolayers. The lysine derivative was transported across the OK cells with a flux rate of 1.37 ± 0.23%/(cm^2 h), which is slightly but not significantly higher than the flux of [^{14}C]mannitol at these cells (1.19 ± 0.08%/(cm^2 h)). Uptake of pyrraline after 2 h was only 0.40 ± 0.05%. Hence, after intestinal absorption in the form of di- or tripeptides, which are degraded to the free amino acids, pyrraline is only poorly reabsorbed in the kidneys and should be excreted via the urine. This finding can easily explain the high excretion rates found in our previous investigations (*15*, *16*).

In conclusion, our study shows that the transepithelial transport of intact Ala-Pyrr, Pyrr-Ala and the flux of free pyrraline across Caco-2 cell monolayers is very low. However, the transepithelial transport of pyrraline at Caco-2 cells is increased up to 15-fold when it is given in the form of dipeptides. Since pyrraline is prone to exist in such peptide form due to its peptidase-inhibitory potential (*6*), its transport is most likely of high nutritional and physiological relevance. In the form of di- or tripeptides, it becomes a substrate for PEPT1 and PEPT2 and is accumulated in the epithelial cells, where the peptides are hydrolyzed very quickly producing high concentrations of free pyrraline. Pyrraline leaves the cell across the basolateral cell membrane by simple diffusion driven by its own gradient or mediated by putative basolateral peptide transporters (*19*). No further barrier exists between this compartment and the bloodstream.

In addition, the study demonstrates the importance of using protein- or peptide-bound substrates instead of free amino acids for the thorough characterization of their intestinal absorption. The transport characteristics of an amino acid such as pyrraline are obviously modulated by the incompleteness of intestinal digestion, whether intrinsic or induced by the antiproteolytic capabilities of the modified amino acids themselves. Further research is therefore needed to assess the impact of protein glycation on their intestinal digestibility. Studies concerning the epithelial transport of other free and peptide-bound Maillard reaction products are currently underway. Furthermore, the results so far show only interaction of Ala-Pyrr and Pyrr-Ala with protein-coupled peptide transporters. Future studies with carrier proteins expressed heterologously will be performed to characterize the specific translocation steps in detail.

ABBREVIATIONS USED

Caco, carcinoma colon; SKPT, spontaneous hypertensive rat kidney proximale tubule; OK, opossum kidney; 3-DG, 3-deoxyglucosulose; AGE, advanced glycation end product; Ala-Pyrr, alanylpyrraline; HPLC, high pressure liquid chromatography; NMR, nuclear magnetic resonance; Pyrr-Ala, pyrralylalanine; TLC, thin layer chromatography.

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