ELSEVIER

Biochimica et Biophysica Acta 1373 (1998) 179–194

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Structural studies of the H^+/oligopeptide transport system from rabbit small intestine

Werner Kramer *, Frank Girbig, Ulrike Bewersdorf, Simone Kohlrautz, Claudia Weyland

Department of Research on Metabolic Diseases, Hoechst Marion Roussel Deutschland GmbH, D-65926 Frankfurt am Main, Germany

Received 15 April 1998; revised 28 May 1998; accepted 5 June 1998

Abstract

A 127-kDa protein was identified as a component of the H^+/oligopeptide transport system in brush-border membrane vesicles from rabbit small intestine by photoaffinity labeling with [^{3}H]cephalexin and further photoreactive β-lactam antibiotics and dipeptides. Reconstitution of stereospecific transport activity revealed the involvement of the 127-kDa protein in H^+-dependent transport of oligopeptides and orally active α-amino-β-lactam antibiotics (Kramer et al., Eur. J. Biochem. 204 (1992) 923–930). H^+-Dependent transport activity was found in all segments of the small intestine concomitantly with the specific labeling of the 127-kDa protein. By enzymatic deglycosylation, fragments of M_r 116 and 95 kDa were obtained from the 127-kDa protein with endoglucosidase F and *N*-glycanase, whereas with endoglucosidase H, a fragment of M_r 116 kDa was formed. These findings indicate that the photolabeled 127-kDa protein is a microheterogenous glycoprotein. Surprisingly, it was found that the solubilized and purified 127-kDa protein showed enzymatic sucrase and isomaltase activity. Inhibition of the glucosidase activities with the glucosidase inhibitor HOE 120 influenced neither H^+/oligopeptide transport nor photoaffinity labeling of the 127-kDa protein. With polyclonal antibodies raised against the purified 127-kDa protein, a coprecipitation of sucrase activity and the photolabeled 127-kDa β-lactam antibiotic binding protein occurred. Target size analysis revealed a functional molecular mass of 165 ± 17 kDa for photoaffinity labeling of the 127-kDa protein, suggesting a homo- or heterodimeric functional structure of the 127-kDa protein in the brush-border membrane. These findings indicate that the H^+/oligopeptide binding protein of M_r 127 000 is closely associated with the sucrase/isomaltase complex in the enterocyte brush-border membrane.

Keywords: Peptide transport; β-Lactam antibiotic; Photoaffinity labeling; Topology; Protein complex; Brush border membrane vesicle; Small intestine; (Rabbit)

1. Introduction

Peptide-derived drugs, such as α-amino-β-lactam antibiotics, angiotensin-converting enzyme inhibitors or renin inhibitors, are taken up in the small intestine by a saturable H^+-dependent active transport system [1–13]. Physiologically this nutrient transport system absorbs small peptides formed by enzymatic hydrolysis of ingested proteins [14–16]. The presence of H^+/oligopeptide transporters in the intestine and the kidney plays an important physiological role in the maintenance of protein nutrition and nitrogen balance. Our laboratory has identified putative protein components of the H^+-dependent uptake system for

* Corresponding author. Fax: +49 (69) 305-13333.

0005-2736/98/$19.00
PII: S0005-2736(98)00103-5

oligopeptides and β-lactam antibiotics in the small intestine and the kidney by photoaffinity labeling, protein purification and reconstitution techniques. In the brush-border membrane of enterocytes from rabbit, rat and pig small intestine, an integral membrane protein of apparent M_r 127 000 was specifically labeled by photoreactive derivatives of penicillins, cephalosporins and dipeptides [5,6,12,13,17–20], whereas in rat renal cortex, two binding proteins of M_r 130 000 and 95 000 were labeled [21,22]. With a different technique (affinity labeling with phenylisothiocyanate in the absence and presence of cephalexin) the specific binding of α-amino-β-lactam antibiotics to a 127-kDa protein was also demonstrated [12]. By purification, immunoprecipitation with polyclonal antibodies [19,23] and by stereospecific reconstitution of transport activity [20], the involvement of the 127-kDa protein in the transport of oligopeptides and peptide-derived drugs across the enterocyte brush-border membrane was demonstrated. Our findings were recently confirmed by publications by Miyazaki et al. Using ceftibuten-affinity chromatography, two proteins of M_r 127 and 117 kDa [24] were purified from rat intestinal brush-border membranes showing H^+-dependent oligopeptide transport after reconstitution in proteoliposomes [24,25].

With a different methodology, other research groups have identified by expression cloning H^+/oligopeptide transporters from the small intestine, PepT1 [26–28] and from the kidney, PepT2 [29,30]. Depending on the cloning strategy used, structurally quite different proteins were identified in small intestine as H^+/oligopeptide transporters, PepT1 [26] by Hediger's group and HPT-1 by Dantzig and coworkers [28]. These two proteins exhibited H^+-dependent uptake of dipeptides and α-amino-β-lactam antibiotics and showed the characteristics of H^+/oligopeptide cotransport in transfected cells. However, both proteins do not show any sequence or structure homology to each other and are thus completely different proteins. The transporter PepT1 has 748 amino acids showing 12 putative transmembrane domains [26] whereas HPT-1 has 832 amino acids with only one transmembrane domain as well as highly conserved regions that are found in the cadherin superfamily [28]. Therefore, three different proteins located in the brush-border membrane of mammalian small intestinal enterocytes were identified exhibiting the characteristics of H^+/oligopeptide cotransport. In the present paper, we describe the further biochemical characterization of the 127-kDa oligopeptide transporter from rabbit small intestine as a component of a protein complex with sucrase/isomaltase.

2. Materials and methods

[Phenyl-4(*n*)-^{3}H]benzylpenicillin (specific radioactivity 8–31 Ci/mmol) was obtained from Amersham (Amersham Buchler, Braunschweig, Germany). [Phenyl-^{3}H]cephalexin (specific radioactivity 1.5 Ci/mmol) was synthesized at Hoechst Aktiengesellschaft by Dr. Raymond Oekonomopulos). D-[U-^{14}C]Glucose (specific radioactivity 258.5 mCi/mmol) and [G-^{3}H]taurocholic acid (specific radioactivity 2.1 Ci/mmol) were from Du Pont de Nemours (NEN Division, Dreieich, Germany). L-Cephalexin was synthesized at Hoechst Aktiengesellschaft. D-Cephalexin, β-lactam antibiotics and marker proteins for the determination of molecular masses in electrophoresis were from Sigma (Munich, Germany). Triton X-100, Triton X-114, *n*-octylglycoside, Serva Blue R 250 and materials for electrophoresis were obtained from Serva (Heidelberg, Germany). Endoglycosidase F, (New England Nuclear, (NEC-150) (EC 3.2.1.96)) and endoglycosidase H (EC 3.2.1.96) was from Sigma (Munich, Germany) whereas *N*-glycanase (EC 3.5.1.52) was from Genzyme (Cambridge, MA). Wheat-germ lectin agarose, wheat-germ lectin Sepharose and Mono-S columns (HR 5/5) were obtained from Pharmacia (Pharmacia LKB Biotechnologie, Freiburg, Germany). Cellulose nitrate filters for transport studies (ME 25; 0.45 μm; 25 mm diameter) were from Schleicher and Schuell (Dassel, Germany) whereas the scintillators Quickszint 501, Quickszint 361, Unisolve I and the tissue solubilizer Biolute S were products of Zinsser Analytic (Frankfurt, Germany). Kits 3359 and 3394 for the determination of the activity of the marker enzymes aminopeptidase N (3359) and γ-glutamyltransferase (3394) and solvents for HPLC were obtained from Merck (Darmstadt, Germany). Protein was determined according to Bradford [31] using the Bio-Rad Kit (Bio-Rad, Munich, Germany). Prefilled columns for HPLC chroma-

tography (Nucleosil 120, 7 μm, C_{18}) were obtained from Macherey-Nagel (Düren, Germany). All other materials were of analytical grade and obtained from the usual commercial sources.

2.1. Preparation of brush-border membrane vesicles

Brush-border membrane vesicles were prepared from the small intestines of male white rabbits by the Mg^{2+}-precipitation method as described previously [5–7,17–21]. The functional integrity of the vesicles was determined by measurement of the Na^+-dependent uptake of D-glucose; after a 15-s incubation, a 25–40-fold overshoot compared to equilibrium was obtained. For the anatomical localization of the H^+/oligopeptide transport system along the duodenum–ileum axis the entire small intestine between the pylorus and the caecum was removed, rinsed with ice-cold sodium phosphate-buffered saline and divided into 8 segments of equal length, numbered 1–8, proximal to distal. From each segment, brush-border membrane vesicles were prepared and the enrichment in the marker enzyme aminopeptidase N (EC 3.4.11.2) was determined. The vesicles were used for transport and labeling experiments immediately after preparation. In all other experiments, jejunal vesicles were prepared from segments 2 to 6.

2.2. Transport measurements

The uptake of cephalexin and D-[U-^{14}C]glucose was measured by the membrane filtration method as described [5–7, 17–21]. The concentration of cephalexin in the incubation mixtures was 2 mM. Uptake studies with D-glucose were performed with 1 μCi D-[U-^{14}C]glucose corresponding to a final concentration of 19.34 μM of D-glucose for each determination and bile acid transport was measured for 1 min with 50 μM (0.75 μCi) [^{3}H]taurocholate. The composition of the incubation media is given in the legends to figures. The transport studies were carried out at 30°C and initiated by mixing of 10 μl of brush-border membrane vesicles (100 μg of protein) with 90 μl of medium containing the substrate. After 1 min of incubation, transport was stopped by the addition of 1 ml of ice-cold stop solution (10 mM Tris-HEPES buffer (pH 7.4)/140 mM KCl) and the suspension was immediately pipetted onto the middle of a prewetted filter. After washing with 5 ml of ice-cold stop solution, the cephalexin taken up by the vesicles was determined by HPLC after extraction of the filters with water, using a Waters HPLC chromatography system (Waters 840 Chromatography Data Station, Waters 490 E Programmable Multiwavelength Detector, WISP Model 712 Sample Processor, Model 510 HPLC pumps). Analysis was performed in a Bischoff 2504 mm column (Bischoff, Leonberg, Germany) filled with LiChrosorb RP 18 (7 μm, Merck, Darmstadt, Germany) using a mixture of solvent A (30 mM sodium phosphate buffer (pH 6.2)/10 mM tetraethylammonium chloride) and solvent B (400 g of solvent A and 468 g of acetonitrile) at a ratio (v/v) of 73% solvent A and 27% solvent B. For the determination of radioactively labeled substrates, the filters were dissolved in 4 ml of scintillator Quickzint 361. Radioactivity was measured by liquid scintillation counting in a Packard TriCarb 2000 counter (Packard, Downers Grove, IL, USA); corrections were made for quenching and eventual chemiluminescence. Each transport experiment was performed at least three times with different membrane preparations. The uptake values for cephalexin are determined in triplicate and are given as mean ± S.D.

2.3. Photoaffinity labeling

For photoaffinity labeling, brush-border membrane vesicles, suspended in 10 mM citrate/Tris buffer (pH 6.0)/140 mM KCl were incubated with the indicated amounts of [^{3}H]benzylpenicillin or [^{3}H]cephalexin for 5 min in the dark. Subsequently, the vesicle suspension was irradiated for 2 min at a wavelength of 254 nm with [^{3}H]benzylenicllin or for 5 min at 300 nm with [^{3}H]cephalexin at 20°C in a Rayonet RPR 100 photochemical reactor equipped with 16 RPR 2530 Å or 16 RPR 3000 Å lamps. After photoaffinity labeling the membrane suspensions were diluted with ice-cold buffer (10 mM sodium phosphate (pH 7.4/300 mM mannitol/4 mM PMSF/4 mM EDTA/4 mM iodacetamide) and subsequently centrifuged at $48\,000 \times g$ for 30 min. The membrane proteins in the resulting pellet were either analyzed by SDS-PAGE after precipitation of proteins or used for chromatography.

2.4. SDS-PAGE and detection of radioactivity

SDS-PAGE was performed on discontinuous SDS slab gels (200×140×1.5 mm) as previously described [5,6,23,32]. The proteins were dissolved in 70 μl of SDS sample buffer (62.5 mM Tris-HCl (pH 6.8)/2% SDS/10% glycerol/5% 2-mercaptoethanol/0.001% Bromophenol blue). After centrifugation at 15 000×*g* for 5 min, the clear supernatants were submitted to SDS-PAGE gel electrophoresis on 7.5–9% gels. After fixing and staining the gels were scanned with a densitometer CD50 (DESAGA, Heidelberg, Germany). For the detection of radioactively labeled polypeptides on SDS gels, the gel tracks were cut into 2-mm slices. After digestion of the polypeptides with 250 μl of Biolute S, 4 ml of scintillator Quickszint 501 was added and radioactivity was measured by liquid scintillation counting using a Tricarb 2000 counter (Packard Instruments).

2.5. Purification of the 127-kDa binding protein for oligopeptides/β-lactam antibiotics

The 127-kDa binding protein for oligopeptides/β-lactam antibiotics was purified from solubilized rabbit jejunal brush-border membrane vesicles after photoaffinity labeling with [^{3}H]benzylpenicillin by wheat-germ lectin chromatography and subsequent cation-exchange chromatography on Mono-S HR 5/5 columns as described [19,23]. The fractions were analyzed for radioactivity, enzymatic activities of aminopeptidase N, sucrase and isomaltase and aliquots were submitted to SDS-PAGE with subsequent determination of the distribution of radioactively labeled 127-kDa protein in the different fractions. The activity of sucrase and isomaltase was determined according to Dahlqvist [33] using sucrose and isomaltose as substrates, respectively.

2.6. Determination between integral and peripheral membrane proteins

To determine whether the photolabeled oligopeptide binding proteins are integral or peripheral membrane proteins, the following investigations were performed using membrane vesicles photolabeled with [^{3}H]benzylpenicillin.

2.6.1. Alkaline extraction

Brush-border membrane vesicles (340 μg of protein) suspended in 300 μl of Tris-HEPES buffer (pH 7.4)/300 mM mannitol were centrifuged at 48 000×*g* for 30 min. The supernatant was discarded, and the pellet was resuspended in 300 μl of 100 mM sodium hydrogen carbonate solution containing 4 mM EDTA and kept at 0°C for 30 min. After centrifugation at 48 000×*g* for 30 min, the supernatant and the pellet were carefully separated, delipidated with chloroform/methanol [23] and submitted to SDS-PAGE.

2.6.2. Solubilization with Triton X-100

Brush-border membrane vesicles (230 μg of protein) were suspended in 200 μl of 10 mM Tris-HEPES buffer (pH 7.4)/300 mM mannitol/0.1% (v/v) Triton X-100 and kept at 0°C for 30 min. After centrifugation at 48 000×*g* for 30 min, both the supernatant and the pellet were delipidated and submitted to SDS-PAGE.

2.6.3. Treatment with Triton X-114

Treatment of membranes with Triton X-114 was performed using a modification of the method described by Bordier [34]. Brush-border membrane vesicles (340 μg of protein) were suspended in 250 μl of 10 mM Tris-HCl buffer (pH 7.3)/1% (v/v) Triton X-114/150 mM NaCl and kept at 0°C for 15 min. After centrifugation at 48 000×*g* for 30 min at 4°C, the supernatant was carefully overlaid to a cushion of 100 μl of 10 mM Tris-HCl buffer (pH 7.3)/6% (w/v) sucrose/150 mM NaCl/0.1% (v/v) Triton X-114 in an Eppendorf tube. After 5 min of incubation at 37°C, the probe was centrifuged at 3000×*g* for 5 min. The upper detergent-poor phase containing hydrophilic proteins was carefully separated from the lower detergent-rich phase containing hydrophobic proteins. Both fractions were diluted with water to a volume of 300 μl, and subsequently the proteins were precipitated as described above and submitted to SDS-PAGE.

2.7. Treatment with deglycosylating enzymes

2.7.1. Neuraminidase (EC 3.2.1.18)

Brush-border membrane vesicles (500 μg of protein) collected after photoaffinity labeling were sus-

pended in 100 μl of 20 mM sodium acetate buffer (pH 5.5)/150 mM NaCl/10 mM $CaCl_2$ and 100 μl (100 mU) of neuraminidase in the above buffer were added. After 2 h incubation at 30°C additional 100 mU of neuraminidase were added and incubation was performed for further 3 h. After dilution with ice-cold 10 mM sodium phosphate buffer (pH 7.4)/4 mM PMSF/4 mM EDTA/4 mM iodoacetamide the vesicles were collected by centrifugation and membrane proteins were analyzed by SDS-PAGE.

2.7.2. Endoglucosidase H (EC 3.2.1.96) and F (EC 3.2.1.96), N-glycanase (EC 3.5.1.52)

Brush-border membrane versicles (500 μg of protein) collected after photoaffinity labeling by centrifugation were dissolved in 100 μl of 1% Triton X-100 and incubated for 30 min at 4°C. After centrifugation at 48 000×*g* for 30 min, the clear supernatant was heated to 90°C for 5 min. After cooling and addition of 100 μl of 100 mM sodium phosphate buffer (pH 6.1)/50 mM EDTA/1% Triton X-100/0.1% SDS/1% 2-mercaptoethanol, 5 μU (5 μl) of en-

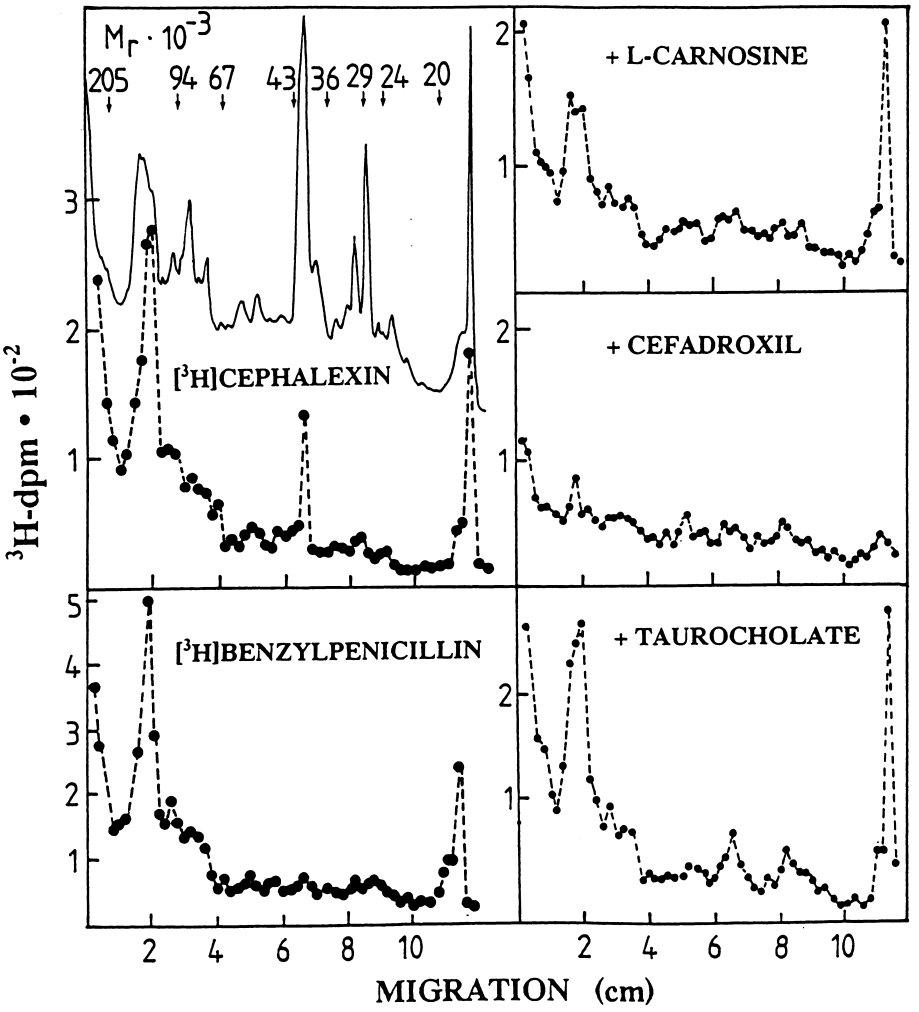

Fig. 1. Direct photoaffinity labeling of rabbit small intestinal brush-border membrane vesicles with [^{3}H]cephalexin. Brush-border membrane vesicles from rabbit small intestine (150 μg of protein) preloaded with 10 mM Tris/HEPES buffer (pH 7.4)/300 mM mannitol were incubated for 5 min in the dark with 6.66 μM (2 μCi) [^{3}H]cephalexin in 10 mM citrate/Tris buffer (pH 6.0)/140 mM KCl in the absence or presence of 250 μM cefadroxil, 2 mM L-carnosine or 250 μM taurocholate and subsequently irradiated at 300 nm for 5 min. For comparison, brush-border membrane vesicles were also labeled with 0.47 μM (2 μCi) [^{3}H]benzylpenicillin. After washing, membrane proteins were separated by SDS-PAGE on 9% gels. After fixing and staining, the distribution of radioactivity was determined by liquid scintillation counting after slicing of the gels into 2-mm slices. The continuous line shows the distribution of Serva Blue R-250-stained polypeptides, the dotted lines show the distribution of radioactivity.

doglucosidase H, 1.7 U (2.5 μl) of endoglucosidase F or 0.9 U (7.2 μl) of *N*-glycanase were added and after 12 h of incubation at 20°C, proteins were precipitated with chloroform/methanol and submitted to SDS-PAGE.

2.8. Target size analysis

Irradiation was performed with high-energy electrons from a 10-MeV linear accelerator at the Strahlenzentrum of the Justus-Liebig Universität, Giessen, Germany. The cryotubes with the ileal brush-border membrane vesicles were placed in the irradiation chamber in front of the linear accelerator. Chamber and samples were cooled with a stream of liquid N_2 flowing through the chamber. The temperature was kept constant in the range of −80 to −130°C, and the actual temperature was measured with an electrode placed within the chamber during irradiation. After irradiation and die-down of induced radioactivity, the samples were stored in liquid N_2 and transported back to the Hoechst laboratories. The following day, the irradiated frozen samples were opened and thawed at 37°C. The vesicles were resuspended by passage through a 27-gauge syringe needle and subsequently placed on ice. Samples of these vesicles were then submitted to photoaffinity labeling. The functional molecular masses were calculated from the empirical equation [35]

$$\log M_r = 5.89 - \log D_{37,T} - 0.0028T$$

where D_{37} is the radiation dosage in Mrad generating 37% of residual biological bio-activity compared with controls and T the temperature (in °C).

3. Results

3.1. [^{3}H]Cephalexin as a direct photoaffinity probe for the H^+/oligopeptide transporter

With photolabile derivatives of penicillins, cephalosporins and dipeptides, a membrane protein of M_r 127 000 was specifically labeled and identified as a prime candidate for the intestinal H^+/oligopeptide transporter [5,6,12,13,17–20]. It was speculated that an α-amino-cephalosporin with a chemical modification of the α-amino function by an 4-azido-[3,5-^{3}H]-benzoyl group would lead to a different labeling pattern compared to the parent α-aminocephalosporin with an intact α-amino function [36]. To investigate this possibility, we have synthesized tritium-labeled cephalexin for direct photoaffinity labeling. Owing to the α,β-unsaturated carbonyl structure and generation of highly reactive radical intermediates via an $n \rightarrow \pi^*$ activation upon irradiation with wavelengths of 300–320 nm cephalosporins can be used for direct photoaffinity labeling of cephalosporin binding proteins as we have shown earlier [12,37]. Photoaffinity labeling of rabbit brush-border membrane vesicles with [^{3}H]cephalexin resulted in an identical labeling pattern as with the hitherto used photoprobes with a specific and prominent labeling of the 127-kDa protein (Fig. 1). Substrates of the H^+/oligopeptide transporter, such as cefadroxil or L-carnosine, were able to inhibit labeling of the [^{3}H]cephalexin binding proteins, whereas taurocholate had no effect. Furthermore, also in brush-border membrane vesicles from rat kidney cortex, identical proteins were labeled by [^{3}H]cephalexin and the other photoaffinity probes (data not shown). These findings clearly indicate that for the identification of putative H^+/oligopeptide transporters in mammalian small intestine and kidney the radioactively labeled substrates benzylpenicillin, cephalexin, 3-azidocephalexin, *N*-(4-azidobenzoyl)-cephalexin and *N*-(4-azidobenzoyl)-glycyl-L-proline are all well suited, since all of them bind to the transporter ligand binding site. For practical reasons concerning the availability of radiolabeled compounds in high amounts and of high specific radioactivity, most experiments described here were performed with [^{3}H]benzylpenicillin. In repeated experiments with the other photoprobes, identical results were obtained.

3.2. Anatomical localization of the H^+/oligopeptide transporter along the duodenum–ileum axis.

To determine the anatomical localization of the H^+/oligopeptide transporter along the duodenum–ileum axis of the small intestine, brush-border membrane vesicles from different regions of rabbit small intestine were prepared to measure H^+/dependent D-cephalexin uptake and to identify the oligopeptide/β-lactam antibiotic binding proteins by photoaffinity labeling. Fig. 2 (upper panel) shows that the

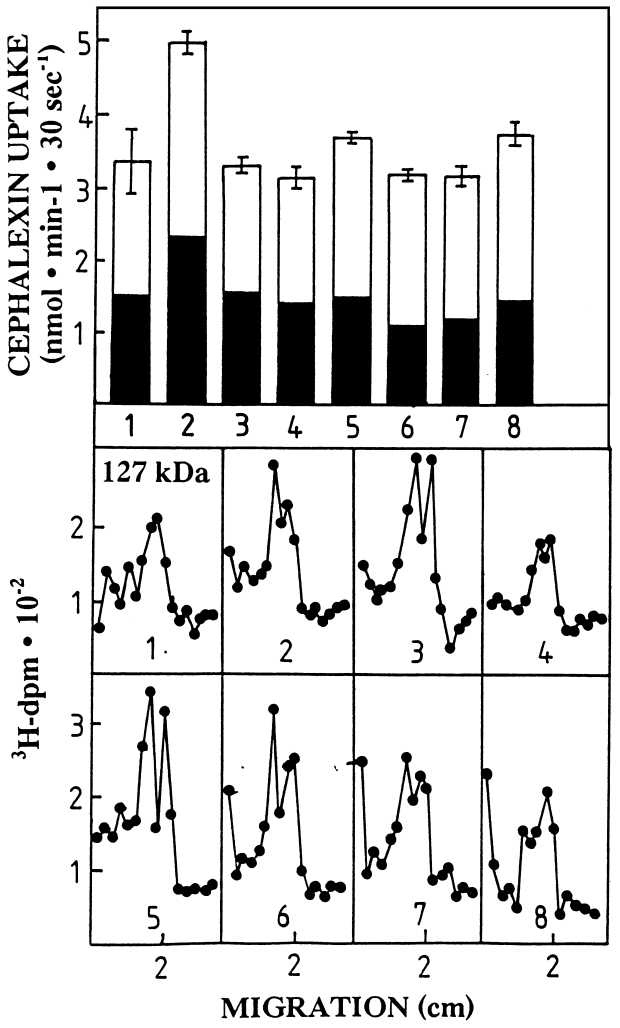

Fig. 2. Anatomical localization of H^+/D-cephalexin transport and photoaffinity labeling of the H^+/oligopeptide transporter along the duodenum–ileum axis in rabbit small intestine. Top: the entire small intestine of rabbits was segmented into 8 pieces of equal length, numbered 1–8, proximal to distal, and brush-border membrane vesicles were prepared. For uptake measurements, brush-border membrane vesicles (100 μg of protein, 10 μl) preloaded with 10 mM Tris/HEPES buffer (pH 7.4)/300 mM mannitol were incubated at 30°C for 30 s with 90 μl either with 2 mM D-cephalexin in 10 mM citrate/Tris buffer (pH 6.0)/140 mM KCl (open bars) or 20 mM Tris/HCl buffer (pH 7.4)/140 mM NaCl (closed bars). Bottom: for photoaffinity labeling, brush-border membrane vesicles (150 μg of protein) of the 8 different small intestinal segments were incubated for 5 min in the dark with 0.47 μM (2 μCi) [^{3}H]benzylpenicillin and subsequently irradiated for 2 min at 254 nm. Afterwards, membrane proteins were separated by SDS-PAGE and the distribution of radioactivity was determined as described in legend to Fig. 1; the numbers indicate the intestinal segments 1–8 from which the brush-border membrane vesicles were prepared. Shown is the distribution of radioactivity in the first 3 cm of each gel track containing the 127-kDa protein.

H^+-dependent transport activity is present in all segments of the small intestine. Corresponding, photoaffinity labeling of the different membrane preparations revealed a specific labeling of the 127-kDa binding protein by [^{3}H]benzylpenicillin in all segments (Fig. 2, lower panels). The labeling of the 127-kDa protein could be inhibited by the orally active cephalosporins cephalexin and cefixime (data not shown). Therefore, the intestinal nutrient transporter for oligopeptides does not show as the D-glucose transporter a strict regional localization to a distinct anatomical site of the small intestine in contrast to the Na^+/bile acid cotransporter [38] or the receptor-mediated uptake system for vitamin B_{12} [39] which are expressed exclusively in the terminal ileum.

3.3. Characterization of the oligopeptide/β-lactam antibiotic binding proteins from rabbit small intestine as integral and peripheral membrane proteins

For a further characterization of the oligopeptide/β-lactam antibiotic binding protein(s) phase separation experiments were performed to discriminate integral from peripheral membrane proteins. It was previously shown that the photolabeled 127-kDa binding protein can be completely solubilized by non-ionic detergents, such as Triton X-100, *n*-octylglucoside or CHAPS [19,23]. By treatment with sodium hydrogen carbonate at pH 10.5, a labeled polypeptide of M_r 120 000 was found in the supernatant, whereas the majority of radioactivity remained in the vesicle pellet (Fig. 3A). By phase separation experiments with Triton X-114, the 127-kDa radiolabeled protein was found in the detergent-rich phase (DRP) indicating a hydrophobic integral membrane protein (Fig. 3B). Interestingly, the majority of radiolabeled proteins was always found in the detergent-poor phase (DPP), possibly a consequence of the hydrophilic nature of the respective proteins due to their microheterogenous glycoprotein structure [23].

3.4. Enzymatic deglycosylation of the binding protein for β-lactam antibiotics/oligopeptides

The broad band of the photolabeled 127-kDa protein on SDS-gels as well as the distribution of the radiolabeled 127-kDa protein on two-dimensional

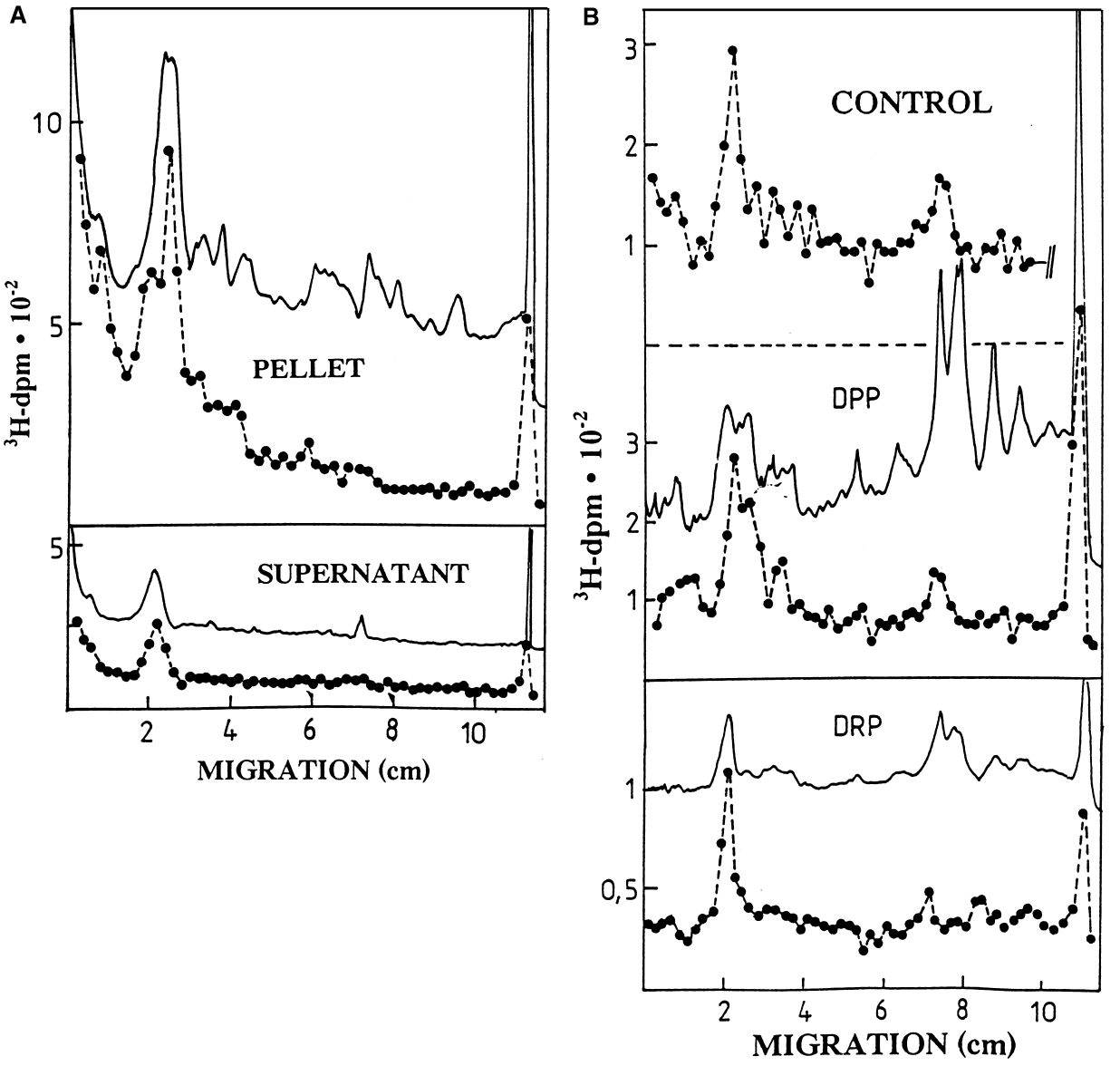

Fig. 3. Phase separation experiments of rabbit small intestinal brush-border membrane vesicles after photoaffinity labeling with [^{3}H]benzylpenicillin. Rabbit small intestinal brush-border membrane vesicles (3.4 mg of protein) preloaded with 10 mM Tris/HEPES (pH 7.4)/300 mM mannitol were incubated for 5 min in the dark with 0.5 μM (30 μCi) [^{3}H]benzylpenicillin in 2.7 ml 10 mM citrate/Tris buffer (pH 6.0)/140 mM KCl and subsequently irradiated for 2 min at 254 nm. After washing, the membrane vesicles were used for the following biochemical examinations. (A) 1 mg of photolabeled brush-border membrane vesicles were suspended in 300 μl of 100 mM sodium hydrogen carbonate solution/4 mM PMSF/4 mM iodoacetamide/4 mM EDTA and kept at 0°C for 30 min. After centrifugation at 48 000×g for 30 min, the proteins in the resulting pellet and supernatant were analyzed by SDS-PAGE on 7.5% gels. (B) 1.2 mg of photolabeled brush-border membrane vesicles were treated with Triton X-114 as described in Section 2 and proteins in control vesicles and the Triton X-114 phases were separated by SDS-PAGE on 9% gels. DRP, detergent-poor phase containing hydrophilic proteins; DPP, detergent-rich phase containing hydrophobic proteins. All other conditions are as described in legend to Fig. 1.

gels over a pH range of about 1 unit, as we have shown previously [23], strongly argues for a microheterogenous glycoprotein. Therefore, both brush-border membrane vesicles or the purified 127-kDa protein were treated with various deglycosylating enzymes. Treatment with neuraminidase had no effect

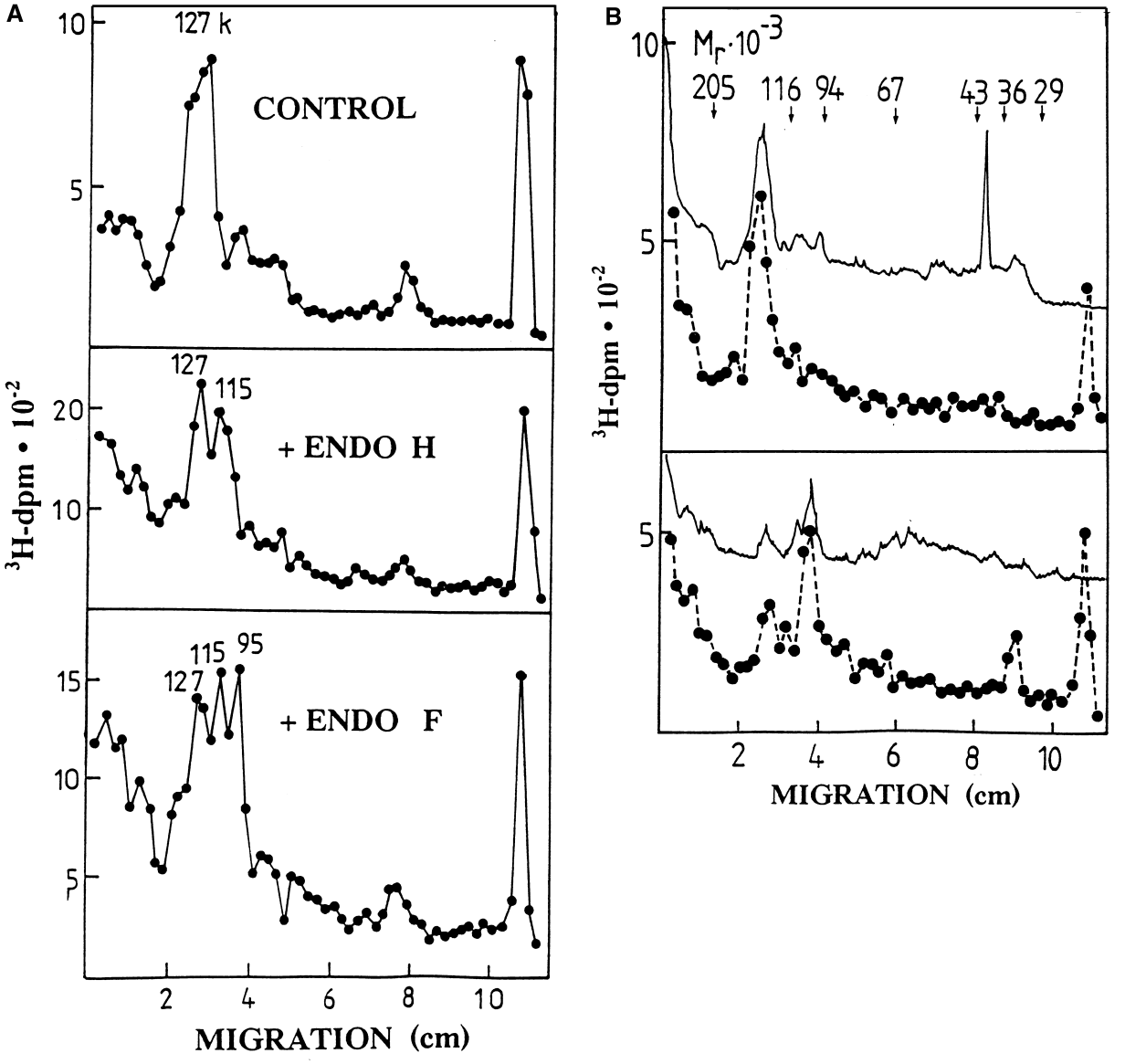

Fig. 4. Enzymatic deglycosylation of rabbit small intestinal brush-border membrane vesicles after photoaffinity labeling with [^{3}H]benzylpenicillin. (A) Rabbit small intestinal brush-border membrane vesicles were photolabeled as described in legend to Fig. 3. A 270 μg amount of labeled membrane proteins was used as control and 670 μg each was submitted to deglycosylation with endoglucosidase H or endoglycosidase F. All other conditions are according to Fig. 1. (B) The purified 127-kDa binding protein for oligopeptides/β-lactam antibiotics (250 μl of eluate from Mono-S column, about 40 μg of protein) was photoaffinity labeled with 0.5 μM (2.5 μCi) [^{3}H]benzylpenicillin. Subsequently proteins were precipitated. The pellet was dissolved in 10 μl of 0.5% SDS/0.1 M 2-mercaptoethanol and 6.5 μl of this solution were submitted to deglycosylation with 7.2 μl (0.9 U) of *N*-glycanase. After SDS-PAGE, the distribution of radioactivity was determined by liquid scintillation counting. The continuous lines shows the densitograms of stained polypeptides, the dotted lines, the distribution of radioactivity. Upper graph: control. Lower graph: after deglycosylation with *N*-glycanase.

on the molecular mass of the 127-kDa protein whereas with endoglycosidases fragments of smaller molecular mass were obtained. With endoglycosidase H a band of M_r 115 000 was formed from the 127-kDa protein, whereas with endoglycosidase F, two radiolabeled bands of M_r 115 000 and 95 000 were formed (Fig. 4A). In addition, the purified, photoaffinity-labeled 127-kDa protein could be degraded to a pro-

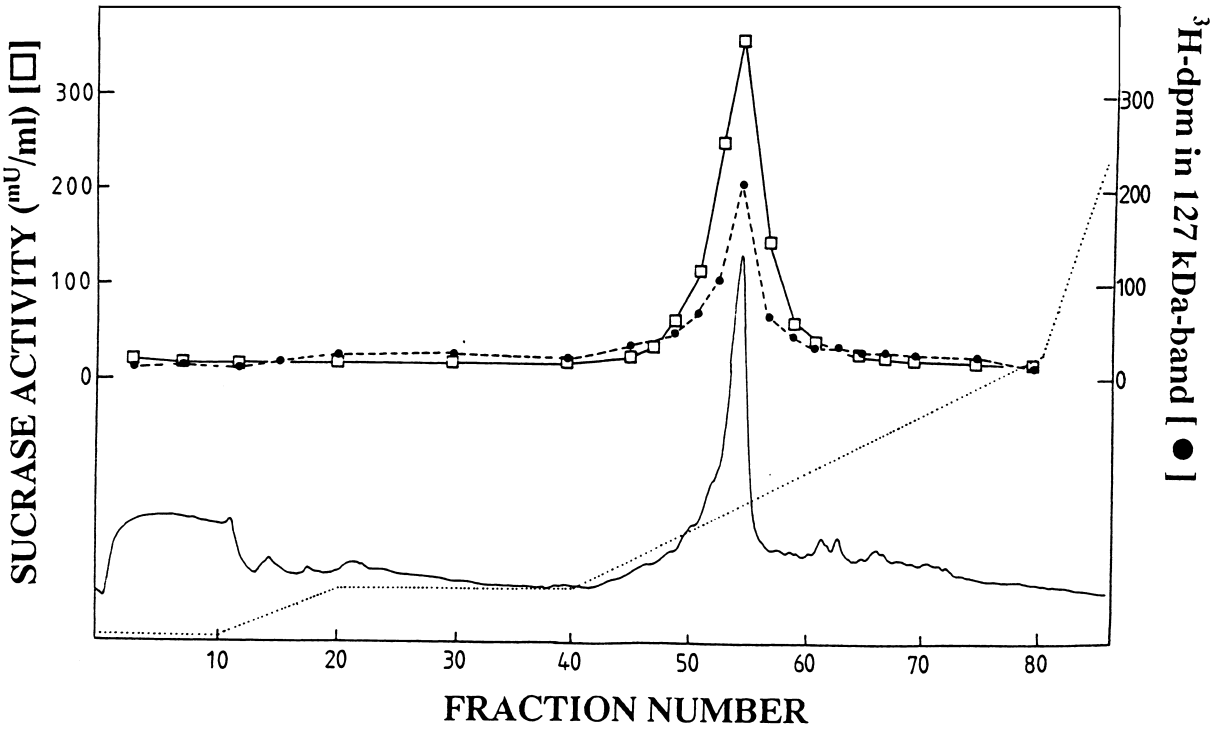

Fig. 5. Purification of the 127-kDa binding protein for oligopeptides/β-lactam antibiotics from rabbit small intestinal brush-border membrane vesicles after photoaffinity labeling with [^{3}H]benzylpenicillin. Rabbit small intestinal brush-border membrane vesicles (3 mg) preloaded with 10 mM Tris/HEPES buffer (pH 7.4)/300 mM mannitol were incubated with 0.83 μM (15 μCi) [^{3}H]benzylpenicillin in 1 ml 10 mM citrate/Tris buffer (pH 6.0)/100 mM mannitol/100 mM KCl for 5 min in the dark at 30°C and subsequently irradiated at 254 nm for 2 min. After washing, the final membrane pellet was suspended in 10 ml of 20 mM sodium acetate buffer (pH 4.5)/0.7% *n*-octyl-glucoside and kept at 20°C for 30 min. After centrifugation, the supernatant was applied to FPLC on a Mono-S HR 5/5 column using 20 mM sodium acetate buffer (pH 4.5)/0.7% *n*-octylglucoside as elution buffer and proteins were eluted by a NaCl-gradient as indicated. Fractions of 1 ml were collected and aliquots were taken to measure the distribution of sucrase activity. From each fraction, 200 μl were removed, proteins were precipitated and submitted to SDS-PAGE on 7.5% gels. The bands containing the 127-kDa protein were excised, proteins were digested with Biolute S and radioactivity was measured by liquid scintillation counting. ——, Absorption at 280 nm; · · · · ·, shape of NaCl gradient; ●-●-●-●, radioactivity in the 127-kDa bands; - - - - -, sucrase activity.

tein of M_r 95–100 000 with *N*-glycanase (Fig. 4B). These studies confirm that the 127-kDa binding protein for oligopeptides/β-lactam antibiotics from rabbit small intestine is a microheterogenous glycoprotein.

3.5. *Copurification of the H$^+$/oligopeptide transport system with the sucrase/isomaltase complex*

The radiolabeled 127-kDa protein appears on SDS gels in a molecular mass range where the ectoenzymes sucrase and isomaltase migrate. It can therefore a priori not be excluded that the photolabeled 127-kDa protein may be related to the sucrase/isomaltase complex. Thus we have purified the radioactively labeled 127-kDa protein from brush-border membrane vesicles which have been photolabeled with [^{3}H]benzylpenicillin and have determined the enzymatic activity of sucrase as well as the distribution pattern of the radiolabeled 127-kDa protein in the individual chromatographic fractions. In wheat germ lectin agarose chromatography as well as in the subsequent cation exchange chromatography on Mono-S columns, the distribution of the radiolabeled 127-kDa protein was parallel to the enzymatic activity of sucrase (Fig. 5), suggesting that the oligopeptide/β-lactam antibiotic binding protein copurifies with the sucrase/isomaltase complex. In contrast, the peptidase aminopeptidase N originally thought to be directly associated with the peptide transporter [40] could be separated from the 127-kDa photolabeled protein under these chromatographic condi-

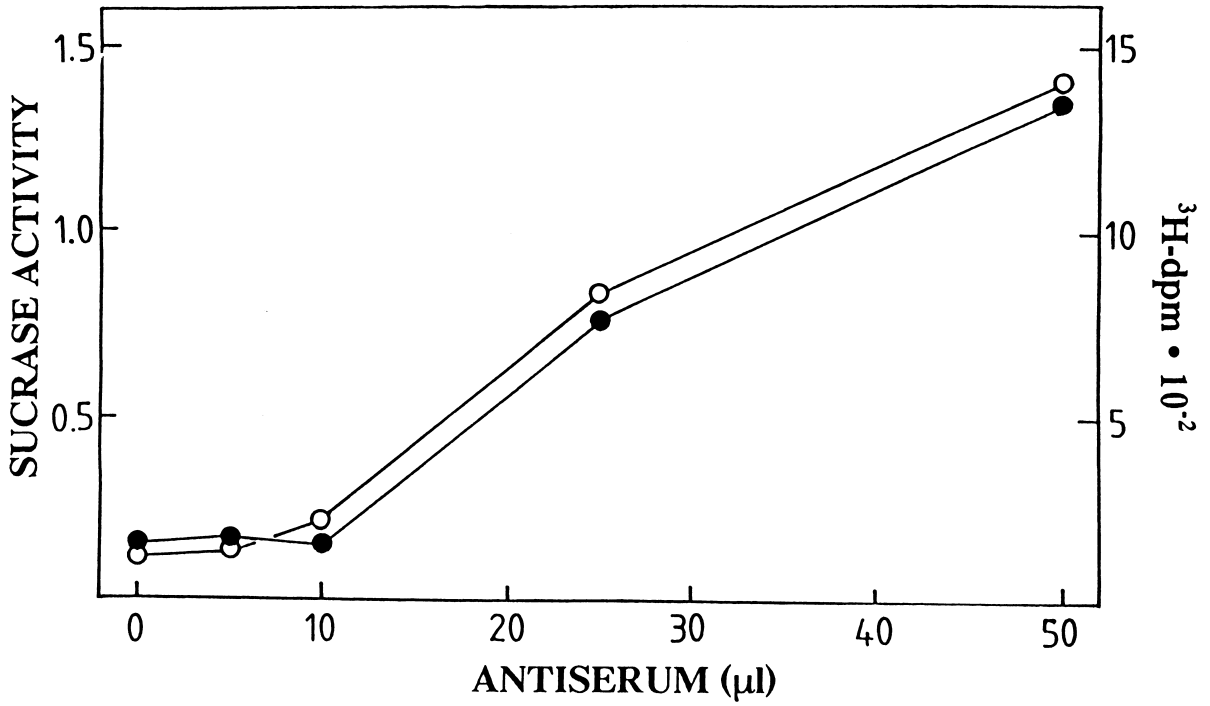

Fig. 6. Immunocoprecipitation of the 127-kDa binding protein for oligopeptides/β-lactam antibiotics with sucrase activity. Rabbit small intestinal brush-border membrane vesicles (1 mg of protein) were photolabeled with 2.2 µM (20 µCi) [^{3}H]benzylpenicillin. After washing, membrane proteins were solubilized by resuspension of the membrane vesicles in 500 µl of 10 mM sodium phosphate buffer/1% Triton X-100 at 4°C for 30 min. After centrifugation and separation of non-solubilized proteins, to 85 µl of supernatant (containing solubilized membrane proteins, 170 µg of protein, 5, 10, 25 or 50 µl of antiserum raised against the purified 127-kDa protein [19,23] were added in a total volume of 250 µl. After 60 min at 4°C, the samples were centrifuged and separated into supernatant and pellet. The pellets were resuspended in 100 µl of 1% Triton X-100. Aliquots of 7 µl from the supernatants and 40 µl aliquots from the solubilized pellet material were used for the determination of sucrase activity and measurement of radioactivity by liquid scintillation counting. ●, Radioactivity in the immunoprecipitate; ○, sucrase activity in the immunoprecipitate.

tions [16]. All attempts to separate the sucrase/isomaltase activity from the photolabeled 127-kDa β-lactam antibiotic binding protein from brush-border membranes solubilized with the non-ionic detergents *n*-octylglucoside or Triton X-100 using a variety of chromatographic procedures [23] failed; all fractions containing the photolabeled 127-kDa protein also contained sucrase/isomaltase activity. Polyclonal antibodies, raised against the purified 127-kDa protein were able to precipitate both, the photolabeled 127-kDa protein and the sucrase activity (Fig. 6). The strict parallelity of radioactively labeled 127-kDa protein and sucrase activity in the immunoprecipitate and the supernatant also strongly suggests a defined protein complex between sucrase/isomaltase and the oligopeptide/β-lactam antibiotic transporter. The 127-kDa protein purified by wheat germ lectin agarose and cation exchange chromatography on Mono-S columns to homogeneity as analyzed by SDS-PAGE could be functionally reconstituted into proteoliposomes exhibiting stereospecific reconstitution of D-cephalexin transport [20]. In these proteoliposomes, sucrase activity could be measured supporting the assumption of a complex between the oligopeptide transporter and sucrase. Chemical crosslinking with bifunctional agents is a valuable tool to identify the neighborhood of proteins and the subunit composition of protein complexes. With bifunctional reagents, such as dimethylsuberimidate, bissuccinimidylsuberate (BSS) or bisulfosuccinimidyl suberate (BS^3), high molecular mass protein products stacking at the start of the concentration gel (gel concentration 4%) or the separation gel (gel concentration 6%) were formed, indicating protein complexes with molecular masses >600 kDa. However, by variation of the pH, the duration of the cross-

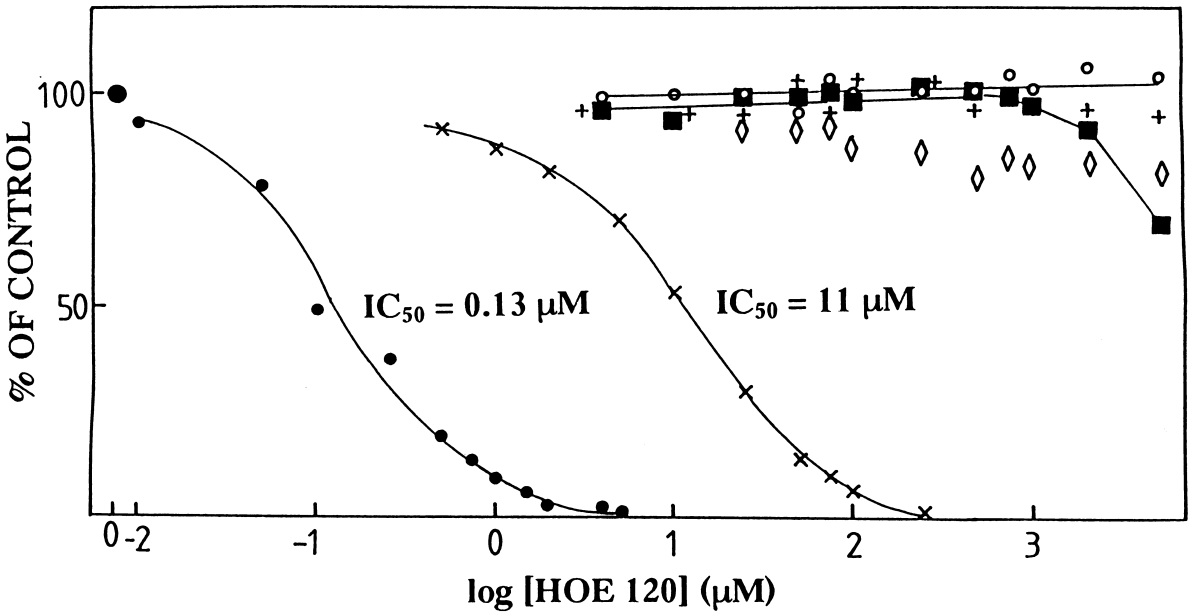

Fig. 7. Effect of the glucosidase inhibitor HOE 120 on the uptake of D-cephalexin, taurocholate and D-glucose and on the enzymatic activity of sucrase, isomaltase and aminopeptidase N. Brush-border membrane vesicles from rabbit small intestine suspended in 10 mM Tris/HEPES buffer (pH 7.4)/300 mM mannitol were incubated with the indicated concentrations of the glucosidase inhibitor HOE 120 and subsequently H^+-dependent D-cephalexin uptake, Na^+-dependent taurocholate and D-glucose transport as well as the enzymatic activities of sucrase, isomaltase and aminopeptidase N were measured as described. The respective activities are expressed as percentage of controls measured in the absence of HOE 120. ●, Sucrase; ×, isomaltase; ○, aminopeptidase N; ■, cephalexin transport; +, taurocholate transport; ◇, D-glucose transport.

linking reaction, the ratio of crosslinker and protein (both, with membrane vesicles and purified 127-kDa protein), the protein complexes with molecular masses between the monomeric 127-kDa protein and the high molecular mass aggregates not could be captured. This suggests a covalent crosslinking between individual protein complexes rather than between the subunits of an individual protein complex.

To investigate a possible direct involvement of sucrase/isomaltase in the transport of oligopeptides/β-lactam antibiotics, the influence of a glucosidase inhibitor, HOE 120, on H^+-dependent uptake of D-cephalexin by small intestinal brush-border membrane vesicles and photoaffinity labeling of the 127-kDa protein was investigated. Fig. 7 shows that the enzymatic activity of sucrase and isomaltase in rabbit brush-border membrane vesicles was concentration-dependently inhibited by HOE 120 with IC_{50} values of 0.13 and 11 μM, respectively. The transport activities of the H^+/oligopeptide transporter, the Na^+-dependent transport systems for bile acids and D-glucose as well as the enzymatic activity of aminopeptidase N were not affected by HOE 120. Photoaffinity labeling of the 127-kDa protein by [^{3}H]benzylpenicillin was also not influenced by the presence of HOE 120 (data not shown). Furthermore, HOE 120 also had no influence on transport or photoaffinity labeling of the H^+/oligopeptide transporter in proteoliposomes, prepared either from solubilized brush-border membrane protein or from the purified 127-kDa protein (data not shown). The findings that the purified 127-kDa protein was homogeneous, according to SDS-PAGE, and that the glucosidase inhibitor HOE 120 completely inhibited sucrase activity with no influence on D-cephalexin transport in proteoliposomes, further supports the assumption that the purified 127-kDa protein exhibits its H^+-dependent oligopeptide transport as well as sucrase/isomaltase activity.

3.6. *Target size analysis of the 127-kDa binding protein for oligopeptides/β-lactam antibiotics*

Since the 127-kDa binding protein for oligopeptides/β-lactam antibiotics is purified as a protein complex, radiation-inactivation studies were performed

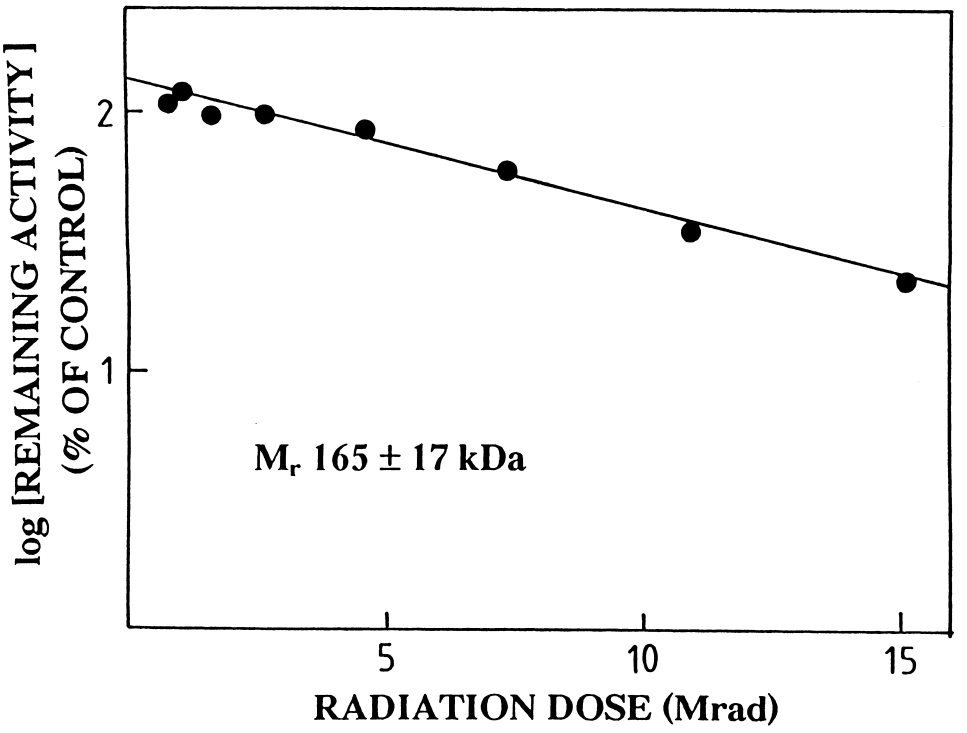

Fig. 8. Target size analysis of photoaffinity labeling of the 127-kDa binding proteins for oligopeptides/β-lactam antibiotics by [^{3}H]benzylpenicillin. Rabbit small intestinal brush-border membrane vesicles equilibrated with 10 mM Tris/HEPES buffer (pH 7.4)/300 mM mannitol/10 mM 2-mercaptoethanol were irradiated with the indicated doses of high-energy electrons. After photoaffinity labeling with 0.44 μM (2 μCi) [^{3}H]benzylpenicillin in 250 μl 10 mM citrate/Tris buffer (pH 6.0)/140 mM KCl membrane proteins were separated by SDS-PAGE and radioactivity in the 127-kDa band was determined by liquid scintillation counting after digestion of proteins with Biolute S. The logarithm of residual labeling (100× (intensity of labeling after irradiation with dose *X*/intensity of labeling of controls)%) of the 127-kDa band was plotted against radiation dose.

to determine the functional molecular mass of the oligopeptide transporter for binding of ligands. Under conditions where, in the irradiated brush-border membrane vesicles, the correct functional molecular mass of the brush-border enzymes aminopeptidase N and sucrase, as well as of the Na^+/D-glucose transporter and the Na^+/taurocholate cotransporter could be determined, no radiation dose-dependence of the H^+-dependent D-cephalexin uptake (2 mM, uptake after 20 s, 60 s and 60 min) could be observed. The reason for this failure remains unclear. In contrast, the functional molecular mass of the H^+/oligopeptide transporter from rat kidney was determined to 416 ± 16 kDa [41]. To identify the functional molecular mass of the 127-kDa binding protein for binding of ligands, photoaffinity labeling with [^{3}H]benzylpenicillin was performed with the radiation-inactivated brush-border membrane vesicles. From six independent experiments, a functional molecular mass of 165 ± 17 kDa (D_{37} value 9.16 ± 0.9 Mrad) was found for the 127-kDa binding protein (Fig. 8). The carbohydrate content of a membrane protein usually does not contribute to the functional molecular mass of a protein or protein complex as was demonstrated by Kempner and coworkers [42]. A molecular mass of 95 kDa was obtained from the photolabeled 127-kDa protein after treatment with endoglycosidase F. Two subunits of M_r 95 kDa would give a molecular mass of 190 kDa which is close to the measured molecular mass of 165 ± 17 kDa.

Interestingly, the binding of bile acids to the 93-kDa subunit of the Na^+/bile acid cotransporter from rabbit ileum [43] as well as to rat liver binding proteins of M_r 48 and 54 kDa [44] also occurs to dimers of the respective proteins. In contrast, for the H^+/oligopeptide transporter from rat kidney the D_{37} values of 3.4 Mrad for the 105-kDa and 2.6 Mrad for

the 130-kDa protein for photoaffinity labeling with [^{3}H]cefadroxil [41] correlate to functional molecular masses >414 kDa, suggesting at least tetrameric structures for the kidney H^+/oligopeptide cotransporter. This different behavior of the intestinal and the kidney H^+/oligopeptide transporter supports our previous conclusions that these transporters are related, but not identical [18,21], a finding recently confirmed by expression cloning of the H^+/peptide cotransporter PEPT 2 from human kidney [29].

4. Discussion

A 127-kDa membrane protein was identified as a component of the H^+/oligopeptide transporter from rabbit small intestine by different approaches: (1) photoaffinity labeling with photoreactive analogs of cephalosporins, penicillins and dipeptides [5,6,12,13,17–20]; (2) in differential photoaffinity labeling studies the 127-kDa polypeptide showed an identical substrate specificity as the transport system [5,6,12]; (3) affinity labeling with phenylisocyanate in the absence and presence of cephalexin [12]; (4) purification of a radiolabeled 127-kDa protein from intestinal brush-border membrane vesicles after photoaffinity labeling of intact membrane vesicles with photoreactive β-lactam antibiotics [19,23]; (5) functional and stereospecific reconstitution of oligopeptide transport activity after preparation of proteoliposomes from the purified 127-kDa protein [19,20]; and (6) affinity chromatography purification of a 127- and a 117-kDa protein using a ceftibuten – matrix and functional reconstitution of H^+-dependent oligopeptide transport activity by incorporation of the purified protein into proteoliposomes [24].

H^+-dependent transport activity was found in the brush-border membrane of enterocytes of the entire small intestine concomitantly with a specific photolabeling of the 127-kDa protein by photoreactive β-lactam antibiotics. By selective extraction and with detergents, it was shown that the 127-kDa band contained two photolabeled proteins of M_r 127 and 120 kDa. By enzymatic deglycosylation radiolabeled proteins of M_r 115 and 95–100 kDa were obtained. Purification of the 127-kDa protein to homogeneity and reconstitution into liposomes revealed that the liposomes containing the 127-kDa protein were able to transport the α-amino-β-lactam antibiotic cephalexin stereospecifically [12,20,23,24]; both enantiomers bind to the transporter [45], but only the D-enantiomer was transported [20], indicating the involvement of the 127-kDa protein in the transport of oligopeptides and peptidomimetics across the intestinal brush-border membrane. An involvement of membrane-bound peptidases for the absorption of peptides either by a multifunctional system or a protein complex has been suggested [40], but no evidence for the involvement of peptidases, such as aminopeptidase N or dipeptidyl-peptidase IV, could be substantiated [16,19]. However, the purified 127-kDa binding protein for oligopeptides and β-lactam antibiotics showed enzymatic activity of the sucrase/isomaltase complex. It was shown that the photolabeled 127-kDa β-lactam antibiotic binding protein copurified with sucrase/isomaltase activity raising the question whether the oligopeptide-transporting 127-kDa protein may be identical to the sucrase/isomaltase complex. A very potent glucosidase inhibitor, HOE 120, inhibited sucrase or isomaltase activity in the μmolar range, but neither had influence on H^+/D-cephalexin cotransport nor on photoaffinity labeling of the 127-kDa protein. Target-size analysis revealed a functional molecular mass of 105 ± 15 kDa for sucrase [43], indicating that the enzymatic activity of sucrase is independent of its tight binding to isomaltase or other proteins. A functional molecular mass of 165 ± 17 kDa for photoaffinity labeling of the 127-kDa protein is suggestive for a homo- or heterodimeric structure of the 127-kDa protein for binding of β-lactam antibiotics.

A dual function of sucrase as an oligosaccharide-degrading enzyme and as a transporter for oligopeptides/β-lactam antibiotics seems improbable. However, the membrane transporters for dibasic and neutral amino acids from rat or rabbit kidney cortex are 78-kDa membrane proteins with only one transmembrane segment showing a high similarity of the extracellular domain with glucosidases [46,47]. Two models for the structure of these transporters are discussed: either an oligomerization to form a functional transporter with multiple transmembrane regions or modulation of the transporter site by a glucosidase-like protein [46]. The strong inhibition of sucrase activity by the disaccharidase inhibitor HOE 120 without any effect on cephalexin transport

or photoaffinity labeling of the 127-kDa protein both in brush-border membrane vesicles as well as in reconstituted proteoliopsomes – makes the latter possibility for the H^+/oligopeptide transporter/sucrase/isomaltase complex unlikely. The relationship of the 127-kDa oligopeptide-binding protein described by us and Miyazaki and coworkers [24,25] to the two structurally completely different H^+/oligopeptide transporters PepT1 and HPT-1 from the small intestine identified by different cloning strategies [26,28,48] remains unclear. The reduction of the molecular masses by deglycosylation to proteins of M_r 78 kDa in the rat [24] and 95 kDa in the rabbit may indicate that the purified 127-kDa protein contains a glycosylated form of the oligopeptide transporter PepT1 which codes for a protein of M_r 79 kDa [24]. Our findings as well as those of Miyazaki and coworkers [24,25] strongly suggest that the H^+/oligopeptide transport system isolated from intestinal brush-border membrane vesicles is isolated as a protein complex composed of several protein species. Preliminary experiments using chemical and enzymatic degradation with the purified 127-kDa protein showed that the 127-kDa protein, being homogeneous on SDS-gels, contained sequence fragments of sucrase, isomaltase and a hitherto unknown protein of M_r 127 kDa being not related to the Pep T1 [26] or HPT-1 transporters [28]. It may well be that a functional transport system isolated from intact tissue has a different subunit composition compared to the transporter functionally expressed by transfection of cells or injection of the respective cDNA into oocytes. Identification of transporters by expression cloning buries the risk, that the mRNA fraction giving the highest signal is amplified leading to the identification of the respective transporter protein. Additional transport systems with lower transport activity may escape their identification. The successful expression cloning of a transporter gives no conclusive information about its physiological contribution and involvement. It is interesting to note that proteins of completely different sequence and structure, but similar or identical function, have been identified in the liver. The Na^+/bile acid cotransporter (NTCP) was identified by expression cloning with ligand uptake measurements in transfected oocytes [49], whereas a Na^+/bile acid cotransporter identical with microsomal epoxide hydrolase was identified by transport inhibition with monoclonal antibodies [50]. Furthermore, a transporter protein expressed in a transgenic cell line, may not fully reflect the behavior of the functional transport system in vivo. For example, the Na^+/D-glucose transporter SGLT1 [51] unambiguously exerts Na^+/D-glucose cotransport. There is, however, increasing evidence that a further 70-kDa protein modulates the activity of SGLT 1 in vivo [52]. Similarly, ileal bile acid absorption is mediated by a 7-transmembrane domain transporter of 348 or 347 amino acids [53]. By different approaches, we could demonstrate that in rabbit ileal brush-border membrane vesicles, the activity of this transporter protein is modulated by interaction with a cytoplasmically attached 14-kDa lipid binding protein [54,55]. Further studies are necessary to identify the role of the 127-kDa β-lactam antibiotic/oligopeptide binding protein and sucrase/isomaltase for H^+-dependent oligopeptide transport in rabbit intestinal brush-border membrane vesicles.

Acknowledgements

The authors thank Mrs. Susanne Winkler for excellent secretarial assistance in preparation of the manuscript.

References

[1] T. Okano, K. Inui, M. Takano, R. Hori, Biochem. Pharmacol. 35 (1986) 1781–1786.
[2] T. Kimura, M. Endo, M. Yoshikawa, S. Muranishi, M. Sezaki, J. Pharmacobio-Dyn. 1 (1978) 262–267.
[3] T. Kimura, T. Yamamato, R. Ishizuka, M. Sezaki, Biochem. Pharmacol. 34 (1985) 81–84.
[4] E. Nakashima, A. Tsuji, H. Mizuo, T. Yamana, Biochem. Pharmacol. 33 (1984) 3345–3352.
[5] W. Kramer, Biochim. Biophys. Acta 905 (1987) 65–74.
[6] W. Kramer, F. Girbig, U. Gutjahr, S. Kowalewski, Biochem. Pharmacol. 46 (1993) 542–546.
[7] W. Kramer, F. Girbig, E. Petzoldt, I. Leipe, Biochim. Biophys. Acta 943 (1988) 288–296.
[8] W. Kramer, F. Girbig, U. Gutjahr, M. Kleemann, I. Leipe, H.-J. Urbach, A. Wagner, Biochim. Biophys. Acta 1027 (1990) 25–30.
[9] H.-W. Kleemann, M. Heitsch, R. Henning, W. Kramer, W. Kocher, U. Lerch, W. Linz, W.-U. Nickel, D. Ruppert, H.-J. Urbach, A. Wagner, R. Weck, F. Wiegand, J. Med. Chem. 35 (1992) 559–567.

[10] U. Hu, G. Amidon, J. Pharm. Sci. 77 (1988) 1007–1011.
[11] S. Yee, G. Amidon, in: M.D. Taylor, G.L. Amidon (Eds.), Peptide-Based Drug Design. Controlling Transport and Metabolism, ACS Professional Reference Book, American Chemical Society, 1995, pp. 135–147.
[12] W. Kramer, F. Girbig, U. Gutjahr, S. Kowalewski, in: M.D. Taylor, G.L. Amidon (Eds.), Peptide-Based Drug Design. Controlling Transport and Metabolism. ACS Professional Reference-Book, American Chemical Society, 1995, pp. 149–179.
[13] W. Kramer, F. Girbig, U. Gutjahr, S. Kowalewski, F. Adam, K.-H. Langer, D. Tripier, W. Schiebler, in: D. Adam, H. Lode, E. Rubinstein (Eds.), Recent Advances in Chemotherapy, Futuramed, Munich, Germany, 1992, pp. 1694–1695.
[14] V. Ganapathy, F.H. Leibach, Am. J. Physiol. 249 (1985) G153–G160.
[15] T. Hoshi, Jpn. J. Physiol. 35 (1985) 179–191.
[16] W. Kramer, D. Dechent, F. Girbig, U. Gutjahr, M. Neubauer, Biochim. Biophys. Acta 1030 (1990) 41–49.
[17] W. Kramer, F. Girbig, I. Leipe, E. Petzoldt, Biochem. Pharmacol. 37 (1988) 2427–2435.
[18] W. Kramer, W. Dürckheimer, F. Girbig, U. Gutjahr, I. Leipe, R. Okonomopulos, Biochim. Biophys. Acta 1028 (1990) 174–182.
[19] W. Kramer, U. Gutjahr, F. Girbig, I. Leipe, Biochim. Biophys. Acta 1030 (1990) 50–59.
[20] W. Kramer, F. Girbig, U. Gutjahr, S. Kowalewski, F. Adam, W. Schiebler, Eur. J. Biochem. 204 (1992) 923–930.
[21] W. Kramer, I. Leipe, E. Petzoldt, F. Girbig, Biochim. Biophys. Acta 939 (1988) 167–172.
[22] W. Kramer, C. Cojocel, D. Mayer, Biochem. Pharmacol. (Life Sci. Adv.) 9 (1990) 127–133.
[23] W. Kramer, F. Girbig, U. Gutjahr, I. Leipe, J. Chromatogr. 521 (1990) 199–210.
[24] K. Iseki, K. Yonemura, T. Kikuchi, I. Naasani, M. Sugawara, M. Kobayashi, N. Kohri, K. Miyazaki, Biochim. Biophys. Acta 1370 (1998) 161–168.
[25] I. Naasani, T. Kikuchi, M. Sugawara, M. Kobayashi, K. Iseki, K. Miyazaki, Biochim. Biophys. Acta 1283 (1996) 185–191.
[26] Y.J. Fei, Y. Kanai, S. Nussberger, V. Ganapathy, F.H. Leibach, F.H. Romero, S.K. Singh, W.F. Boron, M.A. Hediger, Nature 368 (1994) 563–566.
[27] M. Boll, P. Markovich, W.M. Weber, H. Korte, H. Daniel, H. Murer, Pflüger's Arch. Eur. J. Physiol. 429 (1994) 146–149.
[28] A.H. Dantzig, J. Hoskinns, L.B. Tabas, S. Bright, R.L. Shepard, J.L. Jenkins, D.C. Duckworth, J.R. Sportsman, D. Mackensen, P.R. Rosteck Jr., P.L. Skatrud, Science 264 (1994) 430–433.
[29] W. Liu, R. Liang, S. Ramamoorthy, Y.-J. Fei, M.E. Ganapathy, M.A. Hediger, V. Ganapathy, F.H. Leibach, Biochim. Biophys. Acta 1235 (1995) 461–466.
[30] M. Boll, M. Herget, M. Wagner, W.M. Weber, D. Markovich, J. Biber, W. Clauss, H. Murer, H. Daniel, Proc. Natl. Acad. Sci. USA 93 (1996) 284–289.
[31] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[32] G. Burckhardt, W. Kramer, G. Kurz, F.A. Wilson, J. Biol. Chem 258 (1983) 3618–3622.
[33] A. Dahlqvist, Anal. Biochem. 7 (1964) 18–25.
[34] C. Bordier, J. Biol. Chem. 256 (1981) 1604–1607.
[35] R. Béliveau, M. Demeule, H. Ibnoul-Khatib, M. Bergeron, G. Beauvegard, M. Potier, Biochem. J. 252 (1988) 807–813.
[36] H. Daniel, S.A. Adibi, J. Clin. Invest. 92 (1993) 2215–2223.
[37] W. Kramer, Charakterisierung von Transportsystemen für β-Lactam Antibiotika in Plasmamembranen von Darm- und Nierenzellen durch Photoaffinitätsmarkierung. Habilitationsschrift-Johann Wolfgang Goethe-Universität Frankfurt am Main, 1989, pp. 1–200.
[38] W. Kramer, F. Girbig, U. Gutjahr, S. Kowalewski, K. Jouvenal, G. Müller, D. Tripier, G. Wess, J. Biol. Chem. 268 (1993) 18035–18046.
[39] G.J. Russel-Jones, in: M.D. Taylor, G.L. Amidon (Eds.), Peptide-Based Drug Design. Controlling Transport and Metabolism, ACS Professional Reference Book, American Chemical Society, 1995, pp. 181–198.
[40] V. Ganapathy, F.H. Leibach, Life Sci. 30 (1982) 2137–2146.
[41] M. Boll, H. Daniel, Biochim. Biophys. Acta 1233 (1995) 145–152.
[42] E.S. Kempner, J.H. Miller, M.J. McCreery, Anal. Biochem. 156 (1986) 140–146.
[43] W. Kramer, F. Girbig, U. Gutjahr, S. Kowalewski, Biochem. J. 306 (1995) 241–246.
[44] K. Ziegler, R. Elsner, Biochim. Biophys. Acta 1103 (1992) 229–232.
[45] Tsuj, A., in: H. Umezawa (Ed.), Frontiers of Antibiotic Research, Academic Press, New York, 1987, pp. 253–268.
[46] R.G. Wells, M.A. Hediger, Proc. Natl. Acad. Sci. USA 89 (1992) 5596–5600.
[47] J. Bertran, A. Werner, M.L. Moore, G. Stange, D. Markovich, J. Biber, X. Testar, A. Zorzano, M. Palacin, H. Murer, Proc. Natl. Acad. Sci. USA 89 (1992) 5601–5605.
[48] R. Liang, Y.-J. Fei, P.D. Prasad, S. Ramamoorthy, H. Han, T.L. Yang-Feng, M.A. Hediger, V. Ganapathy, F.H. Leibach, J. Biol. Chem. 270 (1995) 6456–6463.
[49] B. Hagenbuch, H. Lübbert, B. Stieger, P.J. Meier, J. Biol. Chem. 265 (1990) 5357–5360.
[50] P. von Dippe, M. Amoni, C. Alves, D. Levy, Am. J. Physiol. 264 (1993) G528–G534.
[51] M.A. Hediger, M.J. Coady, T.S. Ikeda, E.M. Wright, Nature 330 (1987) 379–381.
[52] H. Koepsell, J. Spangenberg, J. Membr. Biol. 138 (1994) 1–11.
[53] M.H. Wong, P. Oelkers, A.L. Craddock, P.A. Dawson, J. Biol. Chem. 269 (1994) 1340–1347.
[54] W. Kramer, F. Girbig, U. Gutjahr, S. Kowalewski, Biochem. J. 306 (1995) 241–246.
[55] W. Kramer, D. Corsiero, M. Friedrich, F. Girbig, S. Stengelin, C. Weyland, Biochem. J. 333 (1998) 335–341.